

**ON MODULATION OF CYCLIC AMP
METABOLISM IN LIVER CELL
MEMBRANES AND FIBROBLASTS
FROM NORMALS AND DIABETICS**

by

Bo Israelsson

Lund 1981.

Malmö 1981



Digitized by the Internet Archive
in 2026

From the Department of Medicine, University of Lund,
Malmö General Hospital, Malmö, Sweden

ON MODULATION OF CYCLIC AMP METABOLISM IN LIVER CELL
MEMBRANES AND FIBROBLASTS FROM NORMALS AND DIABETICS

by

Bo Israelsson



CONTENTS

LIST OF PAPERS	5
ABBREVIATIONS	6
INTRODUCTION	7
GLUCAGON EFFECTS ON PLASMA CYCLIC AMP IN LOW INSULIN RESPONDERS (I)	9
IN VITRO STUDIES ON HUMAN LIVER ADENYLATE CYCLASE (II-IV)	10
Modulation of adenylate cyclase activity by adenosine	10
Modulation of adenylate cyclase activity by zinc ions	11
Adenylate cyclase activity correlated to insulin release and glucose tolerance	12
CHANGES IN ADENYLATE CYCLASE AND 5'-NUCLEOTIDASE ACTIVITIES IN CRUDE LIVER MEMBRANES FROM ALLOXAN DIABETIC RATS (V)	13
CYCLIC AMP ACCUMULATION IN HUMAN SKIN FIBROBLAST CULTURES FROM MATURITY ONSET DIABETICS AND NORMALS (VI)	14
GENERAL SUMMARY	16
ACKNOWLEDGEMENTS	17
REFERENCES	18

LIST OF PAPERS

This thesis is based on the following papers, referred to by Roman numerals in the text. The listed papers can be found in the appendix.

- I GLUCAGON EFFECTS ON PLASMA CYCLIC AMP AND OTHER REACTANTS IN NORMALS AND LOW INSULIN RESPONDERS
Bo Israelsson, Göran Fex, Jörgen Malmquist, and Gunnela Nordén
Acta Med Scand 204(1978)85-87
- II ADENYLATE CYCLASE ACTIVITY IN HUMAN LIVER MEMBRANES AND ITS INHIBITION BY ADENOSINE AND ADENINE NUCLEOTIDES
Bo Israelsson, Anna Berglund, Ulf Ljungqvist, and Jörgen Malmquist
Scand J clin Lab Invest 38(1978)289-293
- III INHIBITION OF HUMAN LIVER MEMBRANE ADENYLATE CYCLASE BY ZINC IONS
Jörgen Malmquist, Bo Israelsson, and Ulf Ljungqvist
Horm Metab Res 11(1979)530-531
- IV ADENYLATE CYCLASE ACTIVITY IN HUMAN LIVER MEMBRANES CORRELATED TO INSULIN RELEASE AND GLUCOSE TOLERANCE
Bo Israelsson
Scand J clin Lab Invest 40(1980)159-162
- V CHANGES IN ADENYLATE CYCLASE AND 5'-NUCLEOTIDASE ACTIVITIES IN LIVER MEMBRANES FROM ALLOXAN DIABETIC RATS
Bo Israelsson and Ingrid Tengrup
EXPERIENTIA 36(1980)257-258
- VI CYCLIC AMP ACCUMULATION IN HUMAN FIBROBLAST CULTURES: NORMALS VERSUS DIABETICS
Bo Israelsson, Jörgen Malmquist, Jan Flink, and Thomas Kjellström
Submitted for publication

ABBREVIATIONS

Cyclic AMP; Adenosine 3',5'-monophosphate

AMP; Adenosine 5'-monophosphate

ADP; Adenosine 5'-diphosphate

ATP; Adenosine 5'-triphosphate

ENZYMES

Adenylate cyclase (EC 4.6.1.1.)

5'-nucleotidase (EC 3.1.3.31.)

INTRODUCTION

Insulin deficiency has long been regarded as the predominant factor in the pathogenesis of diabetes mellitus. However, in a large number of cases, plasma insulin measurements do not indicate a severely reduced secretion. This is particularly true of patients who can be managed without insulin administration, i.e., cases of non-insulin dependent (type 2) diabetes (Reaven and Olefsky, 1978).

In recent years, attention has increasingly been focused on the role of target cell responsiveness in the pathogenesis. Insulin resistance and reduced insulin binding to receptors on mononuclear leukocytes have been documented in type 2 diabetes (Goldstein et al, 1976; Olefsky, 1976; Olefsky and Reaven, 1974). Furthermore, disturbances in intracellular response systems are likely to contribute to insulin resistance and thereby to the diabetic state (Beck-Nielsen, 1978; Olefsky and Reaven 1977). With regard to the possible role of hormonal factors antagonistic to insulin, glucagon (Raskin and Unger, 1978) and epinephrine (Sherwin et al, 1980) have received the most attention.

Blood glucose regulation is heavily dependent on hepatic processes, i.e., glycogen synthesis, glycogenolysis, and gluconeogenesis (Felig, 1975). These metabolic pathways are influenced by levels of cyclic AMP (adenosine 3',5-monophosphate). Insulin may affect these processes by reduction of cyclic AMP (Exton et al, 1971) although other mechanisms may be involved as well. Glucagon, which elevates hepatic glucose output, acts exclusively by the second messenger cyclic AMP, whereas the mechanism of the similar effects of catecholamines is more complex (Exton, 1980).

The cyclic AMP generating system of the plasma membrane, the adenylate cyclase system, consists of at least three subunits (Rodbell, 1980). The mechanism of interaction between the receptor-ligand complex at the outer surface and the enzyme proper at the interior aspect, is not well known (Baxter and Funder, 1979; Pohl, 1977). The complicated build-up results in many possibilities for activity modulation.

The response to hormone stimulation may be modified in at least three different ways: Receptors may be altered, metabolism of second messengers like cyclic AMP may be modulated, and finally possibilities exist for variations in effector systems like intracellular enzymes and transport mechanisms. Insulin resistance is a well known phenomenon in non-insulin dependent diabetics and is related

to a reduction of receptor number (Olefsky, 1976). On the contrary, low levels of plasma insulin with a preservation of normal glucose tolerance has been noticed in so-called low insulin responders (Cerasi et al, 1973). This could be explained by an unusually high insulin sensitivity, possibly related to high number of insulin receptors per cell, although other mechanisms are conceivable. Such a receptor mechanism was originally noticed in Chinese hamsters with genetic insulin deficiency (Hepp et al, 1975). Glucagon receptors may also be subject to changes in number (Tell et al, 1978), and hypothetically glucose homeostasis could be affected by alterations in the activity of the glucagon stimulated adenylate cyclase system of the liver.

This thesis is concerned with some conditions which may affect cyclic AMP metabolism in human liver tissue and cultured fibroblasts. In addition, some studies have been performed with rat liver. Considering the central role of cyclic AMP as a metabolic signal molecule, the presumption was that all influences on tissue cyclic AMP levels are of potential relevance for the understanding of the diabetic disturbances.

GLUCAGON EFFECTS ON PLASMA CYCLIC AMP IN LOW INSULIN RESPONDERS (I)

By stimulation of cyclic AMP synthesis glucagon increases hepatic glucose production and thereby elevates blood glucose. This is achieved by activation of the glycogenolytic and gluconeogenetic processes. In man, hepatocytes are the only target cells for physiological concentrations of glucagon, and after an intravenous glucagon injection plasma cyclic AMP increment predominantly derives from the liver (Liljenquist et al, 1974).

The importance of glucagon in blood glucose regulation has been questioned because of the short-lived elevation of blood glucose after the administration of glucagon (Sherwin et al, 1976). However, others have pointed to the significance even of such brief glucose elevating influences in diabetes (Raskin and Unger, 1978). Reduced rates of glucose entry into muscle and adipose tissue tends to make glucagon induced hyperglycemia more protracted in the diabetic.

The background of our study was the report by Cerasi et al (1973) on "low insulin responders". For unknown reasons they retain glucose tolerance within the normal range although glucose stimulated rise of insulin in peripheral blood is unusually low. Looking for an explanation we examined whether the glucagon stimulated cyclic AMP generating system of the liver had a lower than usual activity.

A pharmacological (0.5 mg) intravenous dose of glucagon was given to 19 healthy men aged 48-50. Eight of these were low insulin responders defined as those having an insulin response below the 5th percentile in either an oral or an intravenous glucose tolerance test. Figures on insulin release after only one glucose load may be rather unreliable (Köbberling et al, 1980). However, low insulin responders exhibited higher plasma glycerol levels, probably indicating a difference in plasma insulin levels on the glucagon stimulus. Glucagon is a potent stimulator of insulin release (Crockford et al, 1966). Insulin is the main inhibitor of lipolysis while glucagon per se is unimportant as a lipolysis stimulating agent in man (Hales et al, 1978). In the present study glucagon injection did not result in any significant difference in plasma insulin levels between low insulin responders and controls. The same pattern with lipolysis more pronounced and no difference in plasma insulin has been noticed in "pre-diabetic" subjects investigated with a physical exercise test known to stimulate mobilization of lipid from fat stores (Nordlander et al, 1973).

Insulin antagonizes the effect of glucagon on formation and release of cyclic

AMP in the perfused liver (Exton et al, 1971). However, this phenomenon was not apparent in the results of Elkeles et al (1977), who investigated plasma cyclic AMP in diabetics and normals after intravenous injections of glucagon (10 µg/kg). In conformity with these last-mentioned authors we found no difference in plasma cyclic AMP between groups. Accordingly, the assumption that glucagon stimulated cyclic AMP generation was reduced in low insulin responders was not supported. However, any subtle difference in cyclic AMP metabolism could have been masked by the large concentrations of glucagon.

IN VITRO STUDIES ON HUMAN LIVER ADENYLATE CYCLASE (II-IV)

The cyclic AMP generating and eliminating systems are complex and under influence of many factors. In vitro methods facilitate the keeping of constant conditions, and in this respect are more informative than in vivo experiments. Studies on human liver tissue have been infrequent, probably because of the difficulties involved in acquiring material (Scott, 1970; Menon et al, 1973; Pecker et al, 1979). In animal studies isolated hepatocytes have often been used for investigations on liver cyclic AMP metabolism. Preparation of human isolated hepatocytes has unfortunately proved impossible. Liver slices, homogenates, and plasma membranes have found use as good alternatives.

Modulation of adenylate cyclase activity by adenosine (II)

It has been proposed that adenosine is a local hormone, similar to prostaglandins, with its effects mainly localized to the organ in which it is produced (Arch and Newsholme, 1978). Adenosine affects adenylate cyclase activity as a stimulant or an inhibitor depending on tissue type and on nucleoside concentration (Fain and Malbon, 1979). In the liver, adenosine has been shown only to inhibit adenylate cyclase activity (Moriwaki and Foà, 1970; Bär and McKenzie, 1973; Londos and Preston 1977; Londos and Wolff, 1977). Phosphodiesterase inhibitors antagonize adenosine stimulatory effects (Londos and Wolff, 1977). Thus, with the use of liver tissue and the presence of 3-isobutyl,1-methylxanthine in the reaction mixture only inhibition of adenylate cyclase activity was to be expected in our study.

Liver specimens were obtained by intraoperative wedge biopsies taken at elective cholecystectomy and a crude liver membrane preparation was made by a simple procedure consisting of homogenisation, centrifugation and filtration steps. Adenosine as well as the two adenine nucleotides AMP and ADP were tested. All three compounds were inhibitors, adenosine being the most potent. Inhibition

by AMP and ADP may in fact, be related to dephosphorylation to adenosine (Londos and Preston, 1977). Adenosine at a concentration of 10^{-3} mol/l antagonized the stimulatory effect of glucagon on the adenylate cyclase activity. Fluoride and guanine nucleotide stimulated enzyme activities were also reduced. The two last-mentioned substances operate upon the transducer function or at the catalytic moiety of the adenylate cyclase system (Hebdon et al, 1978; Rodbell, 1980). The results suggest that the inhibitory influence of adenosine affects one or both of these components of the enzyme system. The lowest adenosine concentration tested, 10^{-5} mol/l was effective only after glucagon stimulation. This is in agreement with the results by Londos and Preston (1977) showing that the presence of glucagon potentiated the inhibitory effects of adenosine on hepatic adenylate cyclase.

There is as yet no evidence that adenosine modulates glucagon stimulated cyclic AMP production in the intact liver. It has been proposed that in intact cells or their environments concentrations of adenosine are insufficient to affect adenylate cyclase (Fain and Shepherd, 1977). On the other hand, Londos and Preston (1977) have pointed out the scarcity of information on the concentrations of adenosine in liver cells or in hepatic extracellular fluid. They found it quite possible that enough adenosine is formed to depress cyclic AMP production.

Modulation of adenylate cyclase activity by zinc ions (III)

Zinc has been of interest in diabetes research because of its intimate relation to insulin in the pancreatic B cells and its presence in many pharmaceutical insulin preparations. It has been shown that zinc increases the binding and decreases the degradation of insulin at liver cells (Arquilla et al, 1978), and recently an insulin-like activity of zinc on lipogenesis in isolated adipocytes has been reported (Coulston and Dandona, 1980).

In early studies by Rall and Sutherland (1962) zinc ions at a concentration of 1.2×10^{-4} mol/l was shown to greatly inhibit the formation of cyclic AMP in a preparation of adenylate cyclase from skeletal muscle or cerebral cortex. In our experimental system with a human crude liver membrane preparation zinc ions were tested as modulators of glucagon and fluoride stimulated adenylate cyclase activity. Micromolar concentrations of zinc inhibited both conditions of adenylate cyclase stimulation. Zinc concentrations were in the same order of magnitude as plasma zinc levels. However, most of plasma zinc is bound to albumin and therefore active in vivo levels are not precisely known. The mechanism by

which zinc interacts with adenylate cyclase is not clear. However, considering the results mentioned above and the release of zinc from the pancreatic B cells into the portal vein, it seems quite possible that these ions may be one of several factors which modulate hormone effects on the liver cell.

Adenylate cyclase activity correlated to insulin release and glucose tolerance (IV)

Insulin reduces intracellular cyclic AMP in liver cells (Exton et al, 1971). This is achieved by phosphodiesterase stimulation (Loten et al, 1978), possibly in combination with inhibition of adenylate cyclase activity. It has previously been reported by Hepp (1972) that streptozotocin diabetes in mice increases glucagon stimulated adenylate cyclase activity in a particle fraction of the liver. This alteration could be normalized if insulin was injected before the sacrifice of the animals. Fluoride stimulated adenylate cyclase activity was not increased by the diabetic state, leading to the assumption that diabetes affected the hormone activation mechanism rather than the enzyme proper.

After informed consent 17 patients, who were going to be operated with elective cholecystectomy, were tested. Glucose stimulated insulin elevation in peripheral blood expressed as 0-40 minutes increment was determined at an oral glucose tolerance test. The assumption was made that these insulin increments were roughly related to the habitual meal-induced insulin secretion peaks of each individual. Liver specimens were obtained according to previous description (II). No complications occurred. The time interval between the determination of insulin secreting capacity and operation was between 15 and 294 days. In three cases the interval was more than 64 days. However, the great proportion of the total variance in glucose concentrations at oral glucose tolerance test over longer periods only represents a random variation (Köbberling et al, 1980). There was a weak negative correlation between plasma insulin increments and glucagon stimulated adenylate cyclase activity in the crude liver membrane preparations. In agreement with Hepp (1972) this result points to the possibility of an enduring inhibitory influence on adenylate cyclase by insulin or some other mechanisms varying in parallel with the insulin concentrations.

There was also a tendency towards a positive correlation between blood glucose at 120 minutes of the glucose tolerance test and adenylate cyclase activity. Fifteen of 17 subjects exhibited blood glucose levels which correlated well with the adenylate cyclase activity. The result is consistent with the role of cyclic AMP as a mediator of blood glucose elevation.

Recently Arner et al (1980) have published a study on an in vitro investigation of the rate of lipolysis in diabetes correlated to blood glucose levels. Glucose concentrations were positively interrelated to basal and catecholamine induced rates of lipolysis. This represents another example of a cyclic AMP dependent process investigated in vitro which correlates to glucose homeostatic processes determined in vivo.

Insulin deficiency leads to generalized changes in cell surface components (Chandramouli and Carter, 1975; Chandramouli, 1977) and may be associated with the results mentioned above. However, other possibilities exist. The adenosine generating enzyme, 5'-nucleotidase, has a reduced activity in the liver in insulin deficient states (Bhathena et al, 1978), and adenosine is an inhibitor of adenylate cyclase in liver tissue (II) and adipocytes (Schwabe et al, 1975). Thus, low insulin or factors associated with low insulin could possibly have resulted in reduced inhibition of adenylate cyclase by diminished adenosine production.

CHANGES IN ADENYLATE CYCLASE AND 5'-NUCLEOTIDASE ACTIVITIES IN CRUDE LIVER MEMBRANES FROM ALLOXAN DIABETIC RATS (V).

In liver, fat, and cartilage adenosine has purely inhibitory effects on adenylate cyclase activity. During chondrocyte maturation an increase in 5'-nucleotidase and a decrease in adenylate cyclase activities have been noticed (Rodan et al, 1977). In hepatocytes from diabetic rats 5'-nucleotidase is reduced to 50% of controls (Bhathena et al, 1978). There are conflicting results concerning the effect of diabetes on glucagon stimulated adenylate cyclase activity in the liver (Hepp, 1972; Yamashita et al, 1980).

We investigated the 5'-nucleotidase and adenylate cyclase activities in liver membranes prepared from 17 alloxan diabetic and 18 control rats. The crude liver membrane preparation was of the same type as has been previously described from human liver (II-IV). The activity of 5'-nucleotidase was reduced in the diabetic animals. In agreement with Hepp but in contrast to most others we found diabetes to be associated with a slight increase in glucagon stimulated adenylate cyclase activity. Different membrane preparation procedures are likely to be an explanation of these divergent results.

Taking all observations from diabetic and normal rats together there was a negative correlation between the two enzyme activities. Theoretically it is quite possible that this relationship is based on the adenylate cyclase inhibi-

ing effect of adenosine, the product of 5'-nucleotidase. A study pointing to an effect of the 5'-nucleotidase activity on adenylate cyclase activity in thymocytes has recently been presented (Dornand et al, 1980). In contrast to hepatocytes these cells exhibit a stimulatory effect of adenosine on adenylate cyclase. The interrelationship was made clear by finding reduced adenylate cyclase activity after 5'-nucleotidase inhibition by lectin.

CYCLIC AMP ACCUMULATION IN HUMAN SKIN FIBROBLAST CULTURES FROM MATURITY ONSET DIABETICS AND NORMALS (VI)

For future studies on the relationship between adenosine producing capacity and cyclic AMP metabolism an in vitro system with intact cells was needed. Unfortunately, techniques are not available for the use of isolated human hepatocytes or hepatocyte cultures. Therefore skin fibroblasts were chosen. These cells possess adenylate cyclase as well as 5'-nucleotidase (Haslam and Goldstein, 1974; Davèze et al, 1978). A most interesting question is whether these cells by any means are able to express the genetic trait for diabetes even after having been cultured in an artificial milieu.

The study on skin fibroblasts concerned the isoproterenol stimulated cyclic AMP accumulation in cultures from non-insulin dependent diabetics and normals. Results from six normals and four diabetics, a total of 269 observations, indicated a clear difference in cyclic AMP metabolism between the two groups. The cells from diabetics displayed cyclic AMP levels twice as high as those of the control cells. Using a prelabelling technique with [3 H]adenine (Butcher, 1978; Kuo and De Renzo, 1969) phosphodiesterase inhibition had to be excluded in order not to restrict nucleotide efflux (Kelly et al, 1978). Thus, differences in the activities of adenylate cyclase and/or phosphodiesterase may have produced this result. In addition, possibilities exist for variation in adenine uptake or ATP pools between the two groups.

A genetic trait for diabetes in db/db mice (a 45% reduction of insulin receptors per cell) can be recognized in skin fibroblasts cultured over many generations (Raizada et al, 1980). Several comparisons of the characteristics of cultured skin fibroblasts from normals and patients with diabetes have been published. Recently Craig et al (1980) reported on an abnormally low percentage of activated glycogen synthase in cultured fibroblasts from diabetics. Our results likewise suggest that fibroblasts cultured from non-insulin dependent diabetics retain a deviation from cells of normals causing an exaggerated cyclic AMP response to isoproterenol. This aberration may be related to the genetic trait

of type 2 diabetes, and, if expressed in all tissues may be operational in the metabolic disturbances of the disease. Sherwin et al (1980) have recently reported on an excessive rise in plasma glucose after epinephrine infusion on insulin dependent diabetics. This was due to an increased liver responsiveness to epinephrine. Such an alteration could be expected if the cyclic AMP response to catecholamines was augmented in similarity to the present data on fibroblasts.

GENERAL SUMMARY

Studies on glucagon effect on plasma cyclic AMP in low insulin responders (I)

In a study of 19 healthy men plasma cyclic AMP was determined after an intravenous injection of 0.5 mg glucagon. Eight subjects were low insulin responders. Their plasma cyclic AMP was not different from that of the controls. Thus, in this group other mechanisms may compensate for the lower insulin levels. It is possible, however, that in vivo studies are inappropriate for the determination of subtle differences in intracellular cyclic AMP metabolism.

Studies on modulation of adenylate cyclase activity in crude liver membranes (II-V)

Glucagon stimulated adenylate cyclase activities in crude liver membranes from 17 healthy subjects were negatively correlated to insulin increments (0-40 min) determined at a preoperative oral glucose tolerance test. (IV). This may indicate an insulin effect on adenylate cyclase activity, or may be the result of other factors varying in parallel with insulin levels.

Insulin deficiency or some other alteration associated with this deficiency, was shown to reduce the activity of 5'-nucleotidase and to increase the glucagon stimulated adenylate cyclase activity in crude liver membranes from alloxan diabetic rats. In the total of 35 rats a negative correlation was apparent between the activities of these two enzymes. This interrelation may have been effected by adenosine, the product of 5'-nucleotidase (V). In human liver membranes adenosine is an inhibitor of adenylate cyclase activity (II).

Zinc ions in physiological concentrations exert considerable influence on the activity of adenylate cyclase in human crude liver membranes (III). Fluoride and glucagon stimulated adenylate cyclase activities are inhibited to a similar extent, indicating an effect of zinc on the interior parts of the plasma membrane.

Studies on cyclic AMP accumulation in skin fibroblast cultures from maturity onset diabetics and normals (VI)

A total of 269 observations were made on isoproterenol stimulated cyclic AMP accumulation in fibroblast cultures from four non-insulin dependent diabetics and six normals. Fibroblasts from the diabetics exhibited cyclic AMP values

twice as high as those of the controls. Studies of this nature may represent a means of investigating metabolic derangements in diabetes with variable environmental influences eliminated.

The diabetes dilemma is not only the dilemma of insulin supply. These studies illustrate that cellular disturbances in diabetes also include cyclic AMP metabolism and that these abnormalities may be an expression of the genetic trait for the disease. Glucose induced insulin increment is related to glucagon stimulated adenylate cyclase activity of the liver, and variations in endogenous factors like zinc and adenosine are of potential relevance as modulators of this enzyme system.

ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to my teacher and colleague at the lab bench, associate professor Jörgen Malmquist, who has given me his generous support and encouragement. I also want to thank professor Bertil Hood for being a never-ceasing well of inspiration. I am grateful to Anna Berglund and Jan Flink for excellent assistance and valuable suggestions. In addition I thank Inga-Lill Torp and Gunvor Nilsson for skilful secretarial work.

This work was supported by grants from the Swedish Medical Research Council (grant No. B81-19X-05702-02), the Medical Faculty, University of Lund, and the Ernhold Lundström Foundation.

REFERENCES

- Arch JRS and Newsholme EA: The control of the metabolism and the hormonal role of adenosine. *Essays Biochem* 14(1978)82-123
- Arner P, Engfeldt P, and Östman J: Blood glucose control and lipolysis in diabetes mellitus. *Acta Med Scand* 208(1980)297-299
- Arquilla ER, Packer S, Tarmas W, and Miyamoto S: The effect of zinc on insulin metabolism. *Endocrinology* 103(1978)1440-1449
- Bär HP and McKenzie SG: On the action of adenosine upon cardiac and other adenylyl cyclases. *Recent Adv Stud Cardiac Struct Metab* 3(1973)311-318
- Baxter JD and Funder JW: Hormone receptors. *N Engl J Med* 301(1979)1149-1161
- Beck-Nielsen H: The pathogenic role of an insulin-receptor defect in diabetes mellitus of the obese. *Diabetes* 27(1978)1175-1181
- Bhathena SJ, Voyles NR, Smith S, and Recant L: Decreased glucagon receptors in diabetic rat hepatocytes. Evidence for regulation of glucagon receptors by hyperglucagonemia. *J Clin Invest* 61(1978)1488-1497
- Butcher RW: Decreased cAMP levels in human diploid cells exposed to cholinergic stimuli. *J Cyclic Nucleotide Res* 4(1978)411-421
- Cerasi E, Wahren J, Luft R, Felig P, and Hendler R: The regulation of splanchnic glucose production in subjects with low insulin response - a compensatory mechanism in prediabetes? *Europ J Clin Invest* 3(1973)193-200
- Chandramouli V and Carter Jr JR: Cell membrane changes in chronically diabetic rats. *Diabetes* 24(1975)257-262
- Chandramouli V, Williams S, Marshall JS, and Carter Jr JR: Cell surface changes in diabetic rats. Studies on lectin binding to liver cell plasma membranes. *Biochim Biophys Acta* 465(1977)19-33
- Coulston L and Dandona P: Insulin-like effect of zinc on adipocytes. *Diabetes* 29(1980)665-667
- Craig JW, Huang LS, and Lerner J: Insulin stimulation of glycogen synthase activity in cultured human fibroblasts from diabetic and control subjects. *Diabetologia* 18(1980)109-113
- Crockford PM, Porte Jr D, Wood Jr FC, and Williams RH: Effect of glucagon on serum insulin, plasma glucose and free fatty acids in man. *Metabolism* 15 (1966)114-122
- Davêze J-L, Di Pietro A, Frappa J, Deloince R, Beaudry Y, and Fontanges R: Etude cinétique de l'activité 5'-nucléotidase membranaire de cellules diploïdes en culture. *C R Acad Sci* 286(1978)1621-1624
- Dornand J, Bonnaïous J-C, and Mani J-C: 5'-nucleotidase - adenylate cyclase relationships in mouse thymocytes. A re-evaluation of the effects of concanavalin A on cyclic AMP levels. *FEBS Lett* 110(1980)30-34

- Elkeles RS, Hambley J, and Frost PG: The effect of insulin treatment on the metabolic response to glucagon in diabetes. *Diabete Metab* 3(1977)35-38
- Exton JH: Mechanisms involved in α -adrenergic phenomena: role of calcium ions in actions of catecholamines in liver and other tissues. *Am J Physiol* 238(1980)E3-E12
- Exton JH, Lewis SB, Ho RJ, Robison GA, and Park CR: The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. *Am N Y Acad Sci* 185(1971)85-99
- Fain JN and Malbon CC: Regulation of adenylate cyclase by adenosine. *Mol Cell Biochem* 25(1979) 143-169
- Fain JN and Shepherd RE: Adenosine, cyclic AMP metabolism, and glycogenolysis in rat liver cells. *J Biol Chem* 252(1977)8066-8070
- Felig P: The liver in glucose homeostasis in normal man and in diabetes. In: *Diabetes: Its physiological and biochemical basis* (ed. J. Vallance-Owen), p. 93, University Park Press, Baltimore 1975
- Goldstein S, Blecher M, Binder R, Perrino PV, and Recant L: Hormone receptors 5. Binding of glucagon and insulin to human circulating mononuclear cells in diabetes mellitus. *Endocr Res Commun* 2(1975)367-376
- Hales CN, Luzio JP, and Siddle K: Hormonal control of adipose-tissue lipolysis. *Biochem Soc Symp* 43(1978)97-135
- Haslam RJ and Goldstein S: Adenosine 3':5'-cyclic monophosphate in young and senescent human fibroblasts during growth and stationary phase in vitro. Effects of prostaglandin E_1 and of adrenaline. *Biochem J* 144(1974)253-263
- Hebdon M, Le Vine III H, Sahyoun N, Schmitges CJ, and Cuatrecasas P: Properties of the interaction of fluoride- and guanylyl-5'-imidodiphosphate-regulatory proteins with adenylate cyclase. *Proc Natl Acad Sci USA* 75(1978)3693-3697
- Hepp KD: Adenylate cyclase and insulin action. Effect of insulin, nonsuppressible insulin-like material, and diabetes on adenylate-cyclase activity in mouse liver. *Eur J Biochem* 31(1972)266-276
- Hepp KD, Langley J, von Funcke HJ, Renner R, and Kemmler W: Increased insulin binding capacity of liver membranes from diabetic Chinese hamsters. *Nature* 258(1975)154
- Kelly LA, Wu C, and Butcher RW: The escape of cyclic AMP from human diploid fibroblasts: general properties. *J Cyclic Nucleotide Res* 4(1978)423-435
- Köbberling J, Kerlin A, and Creutzfeldt W: The reproducibility of the oral glucose tolerance test over long (5 years) and short periods (1 week). *Klin Wochenschr* 58(1980)527-530
- Kuo JF and De Renzo EC: A comparison of the effects of lipolytic and antilipolytic agents on adenosine 3',5'-monophosphate levels in adipose cells as determined by prior labeling with adenine-8- 14 C. *J Biol Chem* 244(1969)2252-2260

- Liljenquist JE, Bomboy JD, Lewis SB, Sinclair-Smith BC, Felts PW, Lacy WW, Crofford OB, and Liddle GW: Effect of glucagon on net splanchnic cyclic AMP production in normal and diabetic men. *J Clin Invest* 53(1974)198-204
- Londos C and Preston MS: Regulation by glucagon and divalent cations of inhibition of hepatic adenylate cyclase by adenosine. *J Biol Chem* 252 (1977)5951-5956
- Londos C and Wolff J: Two distinct adenosine-sensitive sites on adenylate cyclase. *Proc Natl Acad Sci USA* 74(1977)5482-5486
- Loten EG, Assimacopoulos-Jeannett FD, Exton JH, and Park CR: Stimulation of a low Km phosphodiesterase from liver by insulin and glucagon. *J Biol Chem* 253(1978)746-757
- Menon KMJ, Giese S, and Jaffe RB: Hormone- and fluoride- sensitive adenylate cyclases in human fetal tissues. *Biochim Biophys Acta* 304(1973)203-209
- Moriwaki K and Foà PP: Inhibition of rat liver adenylate cyclase by adenosine and adenine nucleotides. *EXPERIENTIA* 26(1970)22
- Nordlander S, Östman J, Cerasi E, Luft R, and Ekelund L-G: Occurrence of diabetic type of plasma FFA and glycerol responses to physical exercise in prediabetic subjects. *Acta Med Scand* 193(1973)9-21
- Olefsky JM: The insulin receptor: Its role in insulin resistance of obesity and diabetes. *Diabetes* 25(1976)1154-1162
- Olefsky JM and Reaven GM: Decreased insulin binding to lymphocytes from diabetic subjects. *J Clin Invest* 54(1974)1323-1328
- Olefsky JM and Reaven GM: Insulin binding in diabetes. Relationships with plasma insulin levels and insulin sensitivity. *Diabetes* 26(1977)680-688
- Pecker F, Duvaldestin P, Berthelot P, and Hanoune J: The adenylate cyclase system in human liver: characterization, subcellular distribution and hormonal sensitivity in normal or cirrhotic adult, and in foetal liver. *Clinical Science* 57(1979)313-325
- Pohl SL: The glucagon receptor and its relationship to adenylate cyclase. *Fed Proc* 36(1977)2115-2118
- Raizada MK, Tan G, Deo R, and Fellows RE: Cells cultured from the diabetic (DB/DB) mouse have a permanent decrease in insulin receptors. *Endocrinology* 107(1980)1652-1655
- Rall TW and Sutherland EW: Adenyl cyclase. The enzymatically catalyzed formation of adenosine 3'5'-phosphate and inorganic pyrophosphate from adenosine triphosphate. *J Biol Chem* 237(1962)1228-1232
- Raskin P and Unger RH: Glucagon and diabetes. *Med Clin North Am* 62(1978)713-722
- Reaven GM and Olefsky JM: The role of insulin resistance in the pathogenesis of diabetes mellitus. *Adv Metab Disord* 9(1978)313-331

- Rodan GA, Bourret LA, and Cuttler LS: Membrane changes during cartilage maturation. Increase in 5'-nucleotidase and decrease in adenosine inhibition of adenylate cyclase. *J Cell Biol* 72(1977)493-501
- Rodbell M: The role of hormone receptors and GTP-regulatory proteins in membrane transduction. *Nature* 284(1980)17-22
- Schwabe U, Ebert R, and Erbiler HC: Adenosine release from fat cells: Effect on cyclic AMP levels and hormone actions. *Adv Cyclic Nucleotide Res* 5(1975)569-584
- Scott RE: Effect of prostaglandins, epinephrine and NaF on human leukocyte, platelet and liver adenylyl cyclase. *Blood* 35(1970)514-516
- Sherwin RS, Fisher M, Hendler R, and Felig P: Hyperglucagonemia and blood glucose regulation in normal, obese and diabetic subjects. *N Engl J Med* 294(1976)455-461
- Sherwin RS, Shamoon H, Hendler R, Saccà L, Eigler N, and Walesky M: Epinephrine and the regulation of glucose metabolism: Effect of diabetes and hormonal interactions. *Metabolism* 29 Suppl 1(1980)1146-1154
- Tell GP, Haour F, and Saez JM: Hormonal regulation of membrane receptors and cell responsiveness: A review. *Metabolism* 27(1978)1566-1591
- Yamashita K, Yamashita S, Yasuda H, Oka Y, and Ogata E: A decreased response of cyclic adenosine monophosphate concentrations to glucagon in liver slices from streptozotocin-induced diabetic rats. *Diabetes* 29(1980)188-192

Glucagon Effects on Plasma Cyclic AMP and other Reactants in Normals and Low Insulin Responders

Bo Israelsson, Göran Fex, Jörgen Malmquist and Gunnela Nordén

From the Departments of Medicine and Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden

ABSTRACT. The metabolic response to i.v. glucagon was evaluated in 11 normal individuals and 8 healthy low insulin responders. Elevations of plasma cyclic AMP and blood glucose were similar in both groups. Accordingly, no indications were seen of differing hepatic responsiveness to glucagon. In contrast, the groups differed in the course of plasma glycerol during the test.

The liver occupies a central position in glucose homeostasis (5). In many cases of diabetes, the impairment of glucose tolerance is caused more by hepatic overproduction of glucose than by diminished tissue utilization (5). Inappropriately high hepatic glucose output is related to disturbances in insulin and glucagon levels (5, 15) and, at least in some cases, to a reduction in hepatic insulin sensitivity (6). Conversely, individuals with normal glucose tolerance despite a reduction of glucose-induced insulin secretion (low insulin responders) may have an increased hepatic insulin sensitivity (2). It is unknown whether some healthy low insulin responders have a decreased responsiveness to glucagon. To date, disturbed glucagon sensitivity has only been found in uremia, where sensitivity is enhanced (16), and in children with low growth hormone production, whose glucagon sensitivity is lowered (7).

Glucagon is believed to exert its effect on the liver by increasing the formation of adenosine 3',5'-monophosphate (cyclic AMP, cAMP) in the hepatocyte (4). The increase in intracellular cAMP is accompanied by an efflux of the molecule. Measurements of plasma cAMP can therefore serve to indicate changes in intracellular levels (1). Most

or all of the plasma cAMP increment caused by glucagon derives from the liver (12, 18).

The present study aimed to elucidate whether healthy low insulin responders differ from completely normal individuals in their metabolic reactions to exogenous glucagon. Plasma cAMP was used to evaluate hepatic responsiveness. Plasma glycerol was measured as an index of adipose tissue lipolysis and glycerol elimination. Furthermore, glucagon-induced insulin secretion was monitored and compared with glucose-induced secretion.

SUBJECTS AND PROCEDURES

Eight low insulin responders and 11 normal individuals were studied. All 19 had a normal oral or i.v. glucose tolerance. Criteria for normality were blood glucose <6.5 mmol/l at 120 min in the oral glucose tolerance test (30 g glucose/m² BSA) or a k -value >0.9 in the i.v. test (25 g/m²). All individuals were healthy normal-weight (actual/ideal weight 0.92–1.04) males aged 48–50. The insulin response was expressed as the sum of plasma insulin levels obtained during the test procedures. A low insulin response was defined as figures below the 5th percentile of response data obtained in a health control survey of the middle-aged male population from which the study subjects were drawn.

In the morning after 10–12 hours fasting the subjects were given 0.5 mg glucagon (Novo Industries, Copenhagen, Denmark) as an i.v. bolus during 30 sec. Venous blood was taken into heparin tubes (for glycerol) and EDTA tubes before glucagon, 5 min after, and at 10 min intervals thereafter.

ASSAY METHODS

Blood glucose was determined with glucose oxidase according to Marks (13). Plasma cAMP was measured by the radioligand technique of Tovey et al. (19), using the assay kit of the Radiochemical Centre, Amersham, Bucks,

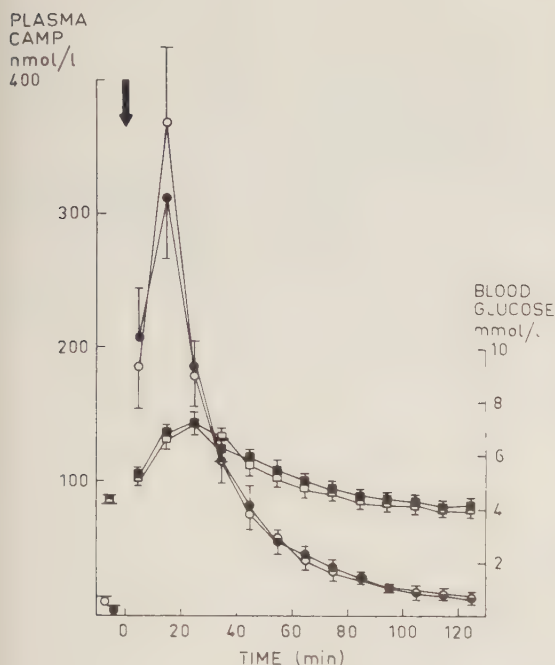


Fig. 1. Effects of glucagon, 0.5 mg i.v., (arrow) on plasma cyclic AMP (●, ○) and blood glucose (■, □) in 8 low insulin responders (filled symbols) and 11 normals (open symbols). Mean $\pm$ S.E.

UK. Plasma glycerol was measured enzymatically by the Laurell and Tibbling procedure (10), and plasma insulin by radioimmunoassay according to Heding (9).

RESULTS

As expected, glucagon injection caused a rapid, marked plasma cAMP increase, which was maximal (300–400 nmol/l) at 15 min post-injection and returned to the initial level (10–20 nmol/l) at 125 min (Fig. 1). Blood glucose rose to a maximum at 25 min and declined to baseline at 125 min (Fig. 1). The plasma cAMP and blood glucose increments in the low insulin responders did not differ from those in the normal individuals.

Likewise, there was no statistically significant difference between groups with regard to insulin response to glucose (Fig. 2).

In all subjects, plasma glycerol decreased during the test. However, at several sampling points glycerol levels were higher in low insulin responders than in normals (Fig. 2).

In all 14 subjects who had had an i.v. glucose tolerance test, the insulin response to glucose was compared with that to glucagon. The total insulin

areas during 5–60 min post-injection were compared. There was no correlation between glucose-induced and glucagon-induced insulin response ($r=0.16$, $p>0.1$).

DISCUSSION

It is believed by many that the primary effect of insulin is to reduce the cAMP-elevating effect of glucagon and other hormones (3). In the present context, however, the glucagon levels obtained were so high that differences between individuals in plasma insulin levels in all probability did not influence hepatic cAMP. Accordingly, the test is seen as an evaluation of hepatic cAMP production capacity at a strong glucagon stimulus. As there was no difference between groups in the responses of cAMP or glucose, no difference was found in hepatic glucagon reactivity.

Plasma glycerol levels reflect adipose tissue lipolysis rates (8). Lipolysis in the rat adipocyte is quite sensitive to the activating effect of glucagon, whereas the human fat cell responds poorly (14). An *in vivo* lipolytic response to glucagon is clearly seen only in subjects with marked insulin deficiency

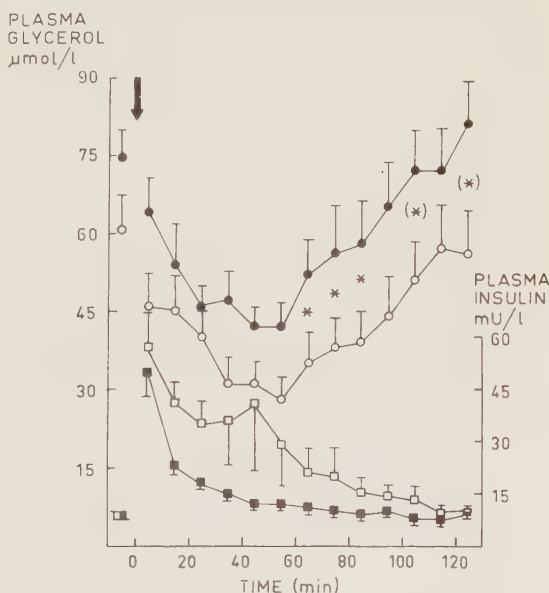


Fig. 2. Effects of glucagon, 0.5 mg i.v., (arrow) on plasma glycerol (●, ○) and plasma insulin (■, □) in 8 low insulin responders (filled symbols) and 11 normals (open symbols). Mean $\pm$ S.E. Differences: * $p<0.05$, (*) $0.05<p<0.10$.

(11). Normal individuals, in contrast, tend to respond by a decrease in plasma glycerol, this being attributed to glucagon-induced insulin release (11).

Interpretation of glycerol data in the present study is difficult. Although differences in insulin levels during the test were not statistically significant, it is possible that the lesser degree of glycerol decline in low insulin responders was a consequence of a lower insulin stimulus on adipose tissue. Furthermore, since pharmacological amounts of glucagon may cause catecholamine release, a difference between the groups in this regard may have influenced lipolysis rates. In addition, the groups may possibly have differed in adipocyte responsiveness to one or more of the hormones. Finally, glycerol levels are influenced by the rate of glycerol removal. It is not known whether any difference in this process exists between the groups studied.

Few data are available on the relation between glucose-induced and glucagon-induced insulin secretion. In accordance with a recent study (17) we found no correlation between the results of these two insulin secretion tests.

Although the present "pharmacologic" dose of glucagon did disclose one slight difference between the groups, a similar test on a lower dose level may conceivably uncover other patterns of difference. Experiments in progress aim at defining a suitably low glucagon dose for further comparisons between normals and low insulin responders.

REFERENCES

1. Broadus, A. E., Hardman, J. G., Kaminsky, N. I., Ball, J. H., Sutherland, E. W. & Liddle, G. W.: Extracellular cyclic nucleotides. *Ann NY Acad Sci* 185: 50, 1971.
2. Cerasi, E., Wahren, J., Luft, R., Felig, P. & Hendler, R.: The regulation of splanchnic glucose production in subjects with low insulin response—a compensatory mechanism in prediabetes? *Eur J Clin Invest* 3: 193, 1973.
3. Exton, J. H., Lewis, S. B., Ho, R. J. & Park, C. R.: The role of cyclic AMP in the control of hepatic glucose production by glucagon and insulin. *Adv Cyclic Nucleotide Res* 1: 91, 1972.
4. Exton, J. H., Robison, G. A., Sutherland, E. W. & Park, C. R.: Studies on the role of adenosine 3',5'-monophosphate in the hepatic actions of glucagon and catecholamines. *J Biol Chem* 246: 6166, 1971.
5. Felig, P.: The liver in glucose homeostasis in normal man and in diabetes. In: *Diabetes: Its physiological and biochemical basis* (ed. J. Vallance-Owen), p. 93. University Park Press, Baltimore 1975.
6. Felig, P., Wahren, J., Hendler, R. & Brundin, T.: Splanchnic glucose and amino acid metabolism in obesity. *J Clin Invest* 53: 582, 1974.
7. Gilbert, P. A. & Wellington, H.: Impaired glucose, insulin, and adenosine, 3',5'-monophosphate responses to glucagon in growth hormone deficient children. *J Clin Endocrinol Metab* 43: 1029, 1976.
8. Havel, R. J.: Some influences of the sympathetic nervous system and insulin on mobilization of fat from adipose tissue: Studies of the turnover rates of free fatty acids and glycerol. *Ann NY Acad Sci* 131: 91, 1965.
9. Heding, L. G.: A simplified insulin radioimmuno assay method. In: *Labelled proteins in tracer studies* (ed. L. Donato, G. Millhaud & J. Sirchis), p. 345. Euratom, Brussels 1966.
10. Laurell, S. & Tibbling, G.: An enzymatic fluorometric micromethod for the determination of glycerol. *Clin Chim Acta* 13: 317, 1966.
11. Liljenquist, J. E., Bomboy, J. D., Lewis, S. B., Sinclair-Smith, B. C., Felts, P. W., Lacy, W. W., Crofford, O. B. & Liddle, G. W.: Effects of glucagon on lipolysis and ketogenesis in normal and diabetic men. *J Clin Invest* 53: 190, 1974.
12. — Effect of glucagon on net splanchnic cyclic AMP production in normal and diabetic men. *J Clin Invest* 53: 198, 1974.
13. Marks, V.: An improved glucose-oxidase method for determining blood, C.S.F. and urine glucose levels. *Clin Chim Acta* 4: 395, 1959.
14. Mitznegg, P., Domschke, W., Domschke, S., Sprügel, W., Estler, C.-J., Wünsch, E., Jaeger, E. & Demling, L.: Effect of glucagon and its 1–23 peptide fragment on lipolysis in isolated rat and human fat cells. *Biochem Pharmacol* 25: 210, 1976.
15. Müller, W. A., Faloona, G. R., Aguilar-Parada, E. & Unger, R. H.: Abnormal alpha-cell function in diabetes. Response to carbohydrate and protein ingestion. *N Engl J Med* 283: 109, 1970.
16. Sherwin, R. S., Bastl, C., Finkelstein, F. O., Fisher, M., Black, H., Hendler, R. & Felig, P.: Influence of uremia and hemodialysis on the turnover and metabolic effects of glucagon. *J Clin Invest* 57: 722, 1976.
17. Solter, M., Vizner, B. & Sekso, M.: The comparison of glucose- and glucagon-induced insulin release in obese and nonobese subjects. *Horm Metab Res* 8: 490, 1976.
18. Strange, R. C. & Mjös, O. D.: The sources of plasma cyclic AMP: Studies in the rat using isoprenaline, nicotinic acid and glucagon. *Eur J Clin Invest* 5: 147, 1975.
19. Tovey, K. C., Oldham, K. G. & Whelan, J. A. M.: A simple direct assay for cyclic AMP in plasma and other biological samples using an improved competitive protein binding technique. *Clin Chim Acta* 56: 221, 1974.

Adenylate cyclase activity in human liver membranes and its inhibition by adenosine and adenine nucleotides

BO ISRAELSSON, ANNA BERGLUND,
ULF LJUNGQVIST & JÖRGEN MALMQUIST

Departments of Medicine and Surgery, University of Lund, Malmö General Hospital,
S-214 01 Malmö, Sweden

Israelsson, B., Berglund, A., Ljungqvist, U. & Malmquist, J. Adenylate cyclase activity in human liver membranes and its inhibition by adenosine and adenine nucleotides. *Scand. J. clin. Lab. Invest.* 38, 289–293, 1978.

The production of adenosine 3',5'-monophosphate (cyclic AMP) in a membrane preparation from human liver homogenate has been studied. Cyclic AMP production was enhanced by glucagon, guanylyl 5'-imidodiphosphate (GMP-PNP), or fluoride, or combinations of these. Adenosine, adenosine monophosphate (AMP) and adenosine diphosphate (ADP) at a concentration of 10^{-3} mol/l antagonized the effects of all stimulants. These data suggest that inhibitory effects are exercised at the catalytic moiety of the adenylate cyclase system, or at the transducer function between hormone receptor and catalytic unit. In contrast, adenosine at a concentration of 10^{-5} mol/l antagonized glucagon- but not fluoride-stimulated adenylate cyclase activity.

Key-words: cyclic AMP; fluoride; glucagon; human liver homogenate

Bo Israelsson, M.D., Department of Medicine, Malmö General Hospital, S-214 01 Malmö, Sweden

Hepatic production of adenosine 3',5'-monophosphate (cyclic AMP, cAMP) is stimulated by glucagon, but declines in continuous presence of the hormone [3]. The mechanism of this limitation of cAMP production is unknown. It is known, however, that concomitantly with cAMP synthesis factors inhibiting adenylate cyclase (E.C. 4.6.1.1) are formed in adipocytes [9, 15]. Adenosine is known to influence cAMP synthesis. In some tissues such as brain [22] and heart [10], and in lymphocytes [26] and platelets [16], adenosine stimulates cAMP formation, whereas the reverse is true in adipose tissue [4, 23] and islets of Langerhans [11]. Adenosine effects on

cAMP synthesis by liver have not been clearly defined, although some data indicate an inhibitory influence [17].

The present study concerns the effects of adenosine, adenosine 5'-monophosphate (AMP) and adenosine 5'-diphosphate (ADP) on the formation of cAMP in human liver tissue. In an attempt to elucidate the site of action of these compounds within the adenylate cyclase complex [21], glucagon and fluoride have been employed as stimulants of cAMP synthesis.

MATERIAL AND METHODS

Chemicals

Protein kinase, protein kinase inhibitor,

cyclic AMP, phosphocreatine, creatine phosphokinase, adenosine, AMP, ADP, and adenosine 5'-triphosphate (ATP) were obtained from Sigma Chemical Co.; 1-methyl, 3-isobutyl-xanthine was from Aldrich Chemical GmbH, Steinheim, G.F.R. Dithiothreitol was from Miles Laboratories, Stoke Poges, U.K. Porcine glucagon was from Novo Industries, Copenhagen, Denmark. Dowex AG 50W-X8 was from Bio-Rad Laboratories, Richmond, California, U.S.A. ^3H -labelled cAMP was obtained from New England Nuclear, Boston, Mass., U.S.A., and guanylyl 5'-imidodiphosphate (GMP-PNP) from Boehringer Mannheim, G.F.R.

Liver biopsy

Liver biopsies, about 1 cm³, were taken during elective cholecystectomies in individuals who were healthy apart from gall-stone disease. The patients gave their consent to the biopsies after information on the purpose and possible risks of the procedure.

The patients had been fasting for 12–18 h. prior to the operation. They received promethazine, pethidine, and atropine, followed by general anaesthesia with pentothal, nitrous oxide, and fentanyl. Succinyl choline was given for muscular relaxation. The liver biopsy was taken as soon as the abdominal cavity had been opened. The technique was that of Gottfries [6], known to entail a minimal risk of complications. Five biopsies were taken for the present studies. No complications occurred.

Preparation of crude membrane fraction from liver homogenate

The tissue was placed in ice cold potassium chloride/potassium acetate buffer, 0.13/0.02 mol/l, pH 7.4, and was minced with scissors. The fragments were homogenized in 10 ml of the same buffer, employing a Polytron PT 10 ST homogenizer for 30 sec at full speed with ice cooling. The homogenate was filtered with suction through three Sartorius membrane filter holders containing nylon cloth of 50 μm porosity. Volume was brought to 40 ml by the addition of ice cold buffer, followed by centrifugation at 48,000 *g* for 15 min at 4°C. The pellet was resuspended in 5 ml of ice cold buffer and the preparation was sucked through another

series of filters of 50, 50, and 25 μm porosity. The preparation which contained 7–10 mg protein per ml, was divided in small aliquots and stored in liquid nitrogen.

Incubation conditions

The incubation medium contained 50 mmol/l Tris-HCl pH 7.6, 2 mmol/l 1-methyl,3-isobutyl-xanthine, 0.1 mmol/l dithiothreitol, 0.1 mmol/l ethylene-diamine-tetraacetic acid (EDTA), 4 mmol/l MnCl_2 , 4 mmol/l ATP, 0.1 mmol/l GMP-PNP, 10 mmol/l phosphocreatine, and 300 $\mu\text{g/ml}$ creatine phosphokinase. To 1.25 ml of medium was added inhibitors (adenosine, AMP, ADP) and either glucagon (final conc. 56 nmol/l) or sodium fluoride (final conc. 10 mmol/l). Total volume was 1.42 ml. Incubation was performed at 37°C in a shaking water bath and the reaction was started by the addition of 50 μl of liver membrane preparation. At 10 min a 1 ml sample was taken and heated at 100°C for 4 min. Zero samples were obtained by adding liver preparation to medium at 100°C.

Cyclic AMP assay

Samples were applied to 7 $\times$ 40 mm columns of AG 50W-X8, 100–200 mesh (hydrogen form, water washed). Columns were washed with 5 ml potassium phosphate 1 mmol/l pH 7.0, followed by 5 ml water [13]. The final 4 ml of eluate was collected. Samples were taken to dryness by air blowing at 37°C, dissolved in sodium acetate buffer pH 4.0, and assayed for cAMP by the method of Gilman [5]. Correction was applied for cAMP loss in the column procedure.

Protein determination

The Hartree modification [7] of the Lowry method was used, with human serum albumin standards.

Results are expressed as nmol cAMP formed per mg protein per 10 min. Student's *t* test was used for statistical analysis.

RESULTS

Cyclic AMP production was found to proceed linearly for the 10 min incubation period employed. The complete incubation mixture did

TABLE I. Adenylate cyclase activity in the presence of various stimulants

Additions	nmol cAMP per mg protein per 10 min	n
None	0.24 ± 0.06	4
Glucagon (56 nmol/l)	0.52 ± 0.14**	4
GMP-PNP (0.1 mmol/l)	1.93 ± 0.40***	4
Glucagon + GMP-PNP	4.76 ± 1.11***	8
Fluoride (10 mmol/l)	1.47 ± 0.59**	4
Fluoride + GMP-PNP	1.60 ± 0.48**	4

Results are means ± SD. Basal cyclic AMP levels (0.01–0.06 nmol/mg protein) have been subtracted. Significance of difference from control incubation: ** $P < 0.01$; *** $P < 0.001$. n = number of experiments.

not display any cAMP phosphodiesterase activity. The formation of cAMP at various incubation conditions is shown in Table I.

Cyclic AMP synthesis in presence of various stimulants

Glucagon increased cAMP production from 1.93 to 4.76 nmol. In the absence of GMP-PNP, basal cAMP synthesis was only 0.24 and glucagon stimulated synthesis 0.52 nmol. On a molar basis, fluoride is much less effective than glucagon as adenylate cyclase stimulant [12, 18, 25]. In the present experiments, cAMP synthesis in the presence of F^- , in medium lacking GMP-PNP, was 1.47 nmol. Fluoride plus GMP-PNP gave a similar result, 1.60 nmol.

TABLE II. Percentage inhibition of cAMP production (stimulated by glucagon + GMP-PNP, fluoride or GMP-PNP) by adenosine, AMP or ADP

Inhibitors	Glucagon + GMP-PNP	Fluoride	GMP-PNP
Adenosine (10 ⁻⁵ mol/l)	54**	0	—
Adenosine (10 ⁻⁴ mol/l)	44**	—	—
Adenosine (10 ⁻³ mol/l)	77***	73**	56**
AMP (10 ⁻⁴ mol/l)	54**	—	—
AMP (10 ⁻³ mol/l)	58***	50*	30*
ADP (10 ⁻⁴ mol/l)	59***	—	—
ADP (10 ⁻³ mol/l)	58***	66**	34**

Significance of inhibitory effect: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Inhibition of cAMP synthesis

The effects of inhibitors are shown in Table II. The presence of 10⁻⁴ mol/l adenosine, AMP or ADP reduced glucagon stimulated cAMP synthesis by 44, 54 and 59% while adenosine, AMP, or ADP, all at 10⁻³ mol/l, reduced synthesis by 77, 58 and 58%, respectively. Adenosine at 10⁻⁵ mol/l was equally effective as 10⁻⁴ mol/l. The effects of inhibitors on fluoride stimulated cAMP synthesis was studied in medium lacking GMP-PNP. Adenosine, AMP and ADP (all 10⁻³ mol/l) reduced cAMP production by 73, 50 and 66%, respectively, while adenosine at the lowest concentration (10⁻⁵ mol/l) had no inhibitory effect. As described above, GMP-PNP was at least as effective as F^- when used as the only stimulant of cAMP production. In these incubations, adenosine, AMP, and ADP (all 10⁻³ mol/l) reduced cAMP formation by 56, 30 and 34%.

Stimulatory and inhibitory effects are sum-

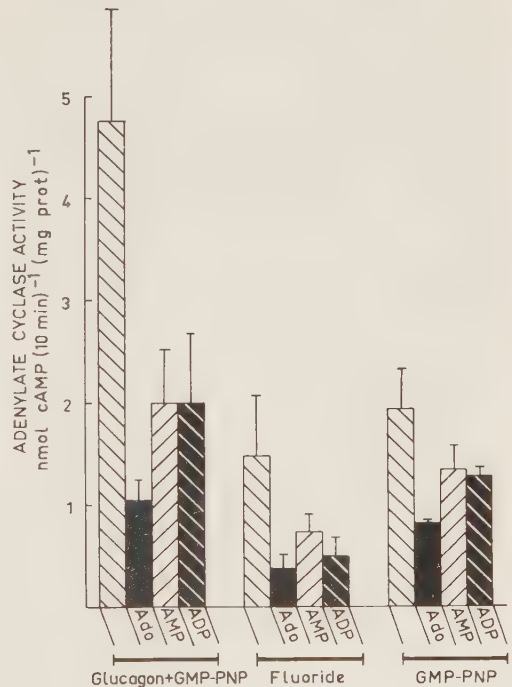


Fig. 1. Adenosine 3',5'-monophosphate (cyclic AMP) production (means and standard deviations) by human liver membrane preparation during incubations with various combinations of stimulatory and inhibitory compounds. Concentrations were: adenosine (ado), adenosine monophosphate (AMP) and adenosine diphosphate (ADP) 1 mmol/l; glucagon 56 nmol/l; fluoride 10 mmol/l and guanylyl 5'-imidodiphosphate (GMP-PNP) 0.1 mmol/l.

marized in Fig. 1 where cyclic AMP production is shown in absolute figures.

DISCUSSION

Liver homogenates and membrane preparations in adenylate cyclase studies

In vitro studies on cAMP formation with normal human liver have not been reported previously. More or less purified rat liver plasma membranes have been employed in most instances [12, 18, 25]. Plasma membranes have a complicated supra-molecular structure [24], and the function of the adenylate cyclase complex is dependent on the maintenance of some elements of *in vivo* membrane topography [14]. While highly purified plasma membrane fragments offer a comparatively well defined material for study, such preparations may conceivably differ in various regards from plasma membranes of the intact cell. The preparations used in the present studies, obtained by simple centrifugation and filtration steps, had rather high hormonal sensitivity and displayed adequately reproducible cAMP formation under various incubation conditions.

Of the multitude of components in homogenate sediments, only plasma membranes synthesize quantitatively important amounts of cAMP according to data from other species [19]. In rat liver, only hepatocyte plasma membrane cAMP synthesis responds to glucagon [25], whereas F^- stimulates adenylate cyclase activity in both hepatocytes and Kupffer cells [25].

Mechanisms of adenylate cyclase stimulation by glucagon and fluoride

Glucagon activates adenylate cyclase by binding to specific receptors on the outer surface of plasma membranes, thereby activating the catalytic moiety of the system on the inside part of the membrane [21]. The mechanism of impulse transduction from outside to inside is unknown. Fluoride ions, in contrast, act directly on the catalytic part of the adenylate cyclase system [1, 20], or possibly on the putative transducer unit located between hormone receptor and the adenylate cyclase proper [8].

The present results confirm previous data from other species [21] in showing the amplifying effect of the GTP analogue GMP-PNP on glucagon

activation of adenylate cyclase. The stimulatory effect of fluoride on the enzyme, in contrast, is minimally influenced by GMP-PNP, which is likewise in accordance with previous reports [12].

The effects of adenosine and adenine nucleotides on cAMP formation

Glucagon-stimulated cAMP formation was markedly reduced by the presence of adenosine, AMP, or ADP. Percentage inhibition by these compounds, at a concentration of 10^{-3} mol/l, was of the same magnitude in experiments with F^- as adenylate cyclase stimulant. The similarities between inhibitions of glucagon and fluoride stimulated cAMP production argue for a site of action of the inhibitors on the catalytic or transducer part of the system. Previous results [17] concerning the inhibitory effects of adenosine, AMP, and ADP (all at 10^{-3} mol/l) on fluoride-stimulated adenylate cyclase activity in rat liver were similar to those obtained in the present work. On the other hand, adenosine at 10^{-5} mol/l only influenced glucagon-stimulated cAMP formation, fluoride-stimulated synthesis being unaffected. Accordingly, at low concentration adenosine would not seem to interact with the catalytic unit. Cyclic AMP synthesis in the presence of GMP-PNP only was inhibited by these compounds to a lesser extent.

During stimulated cAMP production a gradient is formed between intracellular and extracellular cAMP concentrations, the former being considerably higher than the latter [2]. Decomposition products from cAMP may likewise be formed in important amounts at the plasma membrane interior, where they could act as adenylate cyclase inhibitors.

Other sources of adenosine, AMP, and ADP may be of equal or greater importance. These compounds are being formed in cellular ATP turnover, including ATP utilization in cAMP related protein kinase reactions.

Since the extent of dephosphorylation of added nucleotides was not monitored, it is unknown whether the effects of ADP and AMP may be caused by adenosine so produced.

A regulation of adenylate cyclase by hydrolysis products of cAMP and ATP may be of considerable importance in the modulation of hepatocyte metabolism. Attempts to define a possible *in vivo* role of these mechanisms are desirable.

ACKNOWLEDGEMENTS

This study was supported by grants from Nordic Insulin Foundation; Medical Faculty, University of Lund; and the Ernhold Lundström Foundation.

REFERENCES

- Birnbaumer, L., Pohl, S.L. & Rodbell, M. The glucagon-sensitive adenylyl cyclase system in plasma membranes of rat liver. *J. biol. Chem.* **246**, 1857, 1971.
- Christoffersen, T. & Berg, T. Glucagon control of cyclic AMP accumulation in isolated intact rat liver parenchymal cells *in vitro*. *Biochim. biophys. Acta*, **338**, 408, 1974.
- De Rubertis, F.R. & Craven, P. Reduced sensitivity of the hepatic adenylyl cyclase-cyclic AMP system to glucagon during sustained hormonal stimulation. *J. clin. Invest.* **57**, 435, 1976.
- Fain, J.N., Pointer, R.H. & Ward, W.F. Effects of adenosine nucleosides on adenylyl cyclase, phosphodiesterase, cyclic adenosine monophosphate accumulation, and lipolysis in fat cells. *J. biol. Chem.* **247**, 6866, 1972.
- Gilman, A.G. A protein binding assay for adenosine 3',5'-cyclic monophosphate. *Proc. nat. Acad. Sci. (Wash.)*, **67**, 305, 1970.
- Gottfries, A. Studies on acute cholecystitis: A clinical and experimental study in humans and in animals with special reference to liver function and pathogenesis. *Acta chir. scand. Suppl.* **393**, 10, 1968.
- Hartree, E.F. Determination of protein: A modification of the Lowry method that gives a linear photometric response. *Analyt. Biochem.* **48**, 422, 1972.
- Harwood, J.P. & Rodbell, M. Inhibition by fluoride ion of hormonal activation of fat cell adenylyl cyclase. *J. biol. Chem.* **248**, 4901, 1973.
- Ho, R.-J., Russell, T.R., Asawaka, T. & Sutherland, E.W. Cellular levels of feedback regulator of adenylyl cyclase and the effect of epinephrine and insulin. *Proc. nat. Acad. Sci. (Wash.)*, **72**, 4739, 1975.
- Huang, M. & Drummond, G.I. Effect of adenosine on cyclic AMP accumulation in ventricular myocardium. *Biochem. Pharmacol.* **25**, 2713, 1976.
- Ismail, N.A., El Denshay, E.-E. S.M. & Montague, W. Adenosine and the regulation of insulin secretion by isolated rat islets of Langerhans. *Biochem. J.* **164**, 409, 1977.
- Johnson, R.A., Pilkis, S.J. & Hamet, P. Liver membrane adenylyl cyclase. *J. biol. Chem.* **250**, 6599, 1975.
- Kuo, J.-F. & Greengard, P. An assay method for cyclic AMP and cyclic GMP based upon their abilities to activate cyclic AMP-dependent and cyclic GMP-dependent protein kinases. *Advanc. cyclic Nucl. Res.* **2**, 41, 1972.
- Levey, G.S. The role of phospholipids in hormone activation of adenylyl cyclase. *Rec. Progr. hormone Res.* **29**, 361, 1973.
- Manganiello, V.C., Murad, F. & Vaughan, M. Effects of lipolytic and antilipolytic agents on cyclic 3',5'-adenosine monophosphate in fat cells. *J. biol. Chem.* **246**, 2195, 1971.
- Mills, D.C.B. & Smith, J.B. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3',5'-cyclic monophosphate in platelets. *Biochem. J.* **121**, 185, 1971.
- Moriwaki, K. & Foà, P.P. Inhibition of rat liver adenylyl cyclase by adenosine and adenine nucleotides. *Experientia*, **26**, 22, 1969.
- Pohl, S.L., Birnbaumer, L. & Rodbell, M. The glucagon-sensitive adenylyl cyclase system in plasma membranes of rat liver. *J. biol. Chem.* **246**, 1849, 1971.
- Posternak, T. Formation and metabolism of cyclic AMP. p 72 in Robison, G.A., Butcher, R.W. & Sutherland, E.W. (eds.) *Cyclic AMP*. Academic Press, New York, 1971.
- Rodbell, M., Birnbaumer, L. & Pohl, S.L. Adenylyl cyclase in fat cells. *J. biol. Chem.* **245**, 718, 1970.
- Rodbell, M., Lin, M.C., Saloman, Y., Londos, C., Harwood, J.P., Martin, B.R., Rendell, M. & Berman M. The role of adenine and guanine nucleotides in the activity and response of adenylyl cyclase systems to hormones. Evidence for multi-site transition sites. *Acta Endocr. (Kbh.)* **77**, Suppl. 191, 11, 1973.
- Sattin, A., Rall, T.W. & Zanella, J. Regulation of cyclic adenosine 3',5'-monophosphate levels in guinea-pig cerebral cortex by interaction of alpha adrenergic and adenosine receptor activity. *J. Pharmacol. exp. Ther.* **192**, 22, 1975.
- Schwabe, U., Ebert, R. & Erbiler, H.C. Adenosine release from fat cells: Effects on cyclic AMP levels and hormone actions. *Advanc. cyclic Nucl. Res.* **5**, 569, 1975.
- Singer, S.J. & Nicolsen, G.L. The fluid mosaic model of the structure of cell membranes. *Science*, **175**, 720, 1972.
- Wincek, T.J., Hupka, A.L. & Sweet, F.W. Stimulation of adenylyl cyclase from isolated hepatocytes and Kupffer cells. *J. biol. Chem.* **250**, 8863, 1975.
- Wolberg, G., Zimmerman, T.P., Hiemska, W., Winston, M. & Chu, L.-C. Adenosine inhibition of lymphocyte-mediated cytotoxicity: possible role of cyclic adenosine monophosphate. *Science*, **187**, 957, 1975.

Received 14 September 1977

Accepted 12 January 1978

Stauffacher, W., A.E. Lambert, D. Vecchio, A.E. Renold: *Diabetologia* 3: 230–237 (1967)
 Van den Brande, J.L., S. Van Buul, U. Heinrich, F. Van Roon, T. Zurcher, A.C. Van Steirtegem: *Adv. Metab. Dis.* 8: 171–181 (1975)

Van Wyk, J.J., L.E. Underwood, R.C. Lister, R.N. Marshall: *Amer. J. Dis. Childhd.* 126: 705–711 (1973)

Requests for reprints should be addressed to: G.S.G. Spencer, A.R.C., Meat Research Institute, Langford, Bristol (United Kingdom)

Horm. Metab. Res. 11 (1979) 530–531

Inhibition of Human Liver Membrane Adenylate Cyclase by Zinc Ions

J. Malmquist, B. Israelsson and U. Ljungqvist

Departments of Medicine and Surgery, University of Lund, Malmö General Hospital, Malmö, Sweden

The liver occupies a central position in the regulation of glucose metabolism. Hormonal and other stimuli impinging on the hepatocyte influence hepatic glucose uptake and output. A large body of evidence indicates that glucagon enhances glucose production by stimulating the enzyme complex adenylate cyclase in the hepatocyte plasma membrane, thereby increasing intracellular levels of adenosine 3',5'-monophosphate (cAMP) (Exton, Lewis, Ho, Robison and Park 1971). Conversely insulin action may be related to a lowering of cAMP concentration, although this important question is still a matter of discussion (Czech 1977).

Understanding of hormonal regulation requires that the responsiveness (or resistance) of target cells be taken into account. Endogenous nonhormonal factors that influence hepatic adenylate cyclase reactivity deserve attention as possible modulators of glucose homeostasis in the normal and/or diabetic state.

Materials and Methods

Liver biopsies (about 1 cm³) were obtained during cholecystectomies on patients who were healthy apart from gallstone disease. Informed consent was received from the patients. No complications occurred. Six biopsies were used in the present study. Crude membrane preparations were obtained by homogenization, filtration, and centrifugation, as described previously (Israelsson et al. 1978). Incubations were performed in the following medium: 50 mM Tris-HCl pH 7.6, 2 mM 1-methyl, 3-isobutylxanthine, 4 mM MnCl₂, 4 mM ATP, 0.1 mM guanylyl imidodiphosphate, 10 mM phosphocreatine, 300 µg/ml creatine kinase, 0.1 mM EDTA, and 0.1 mM dithiothreitol. Adenylate cyclase was stimulated by glucagon (56 nM) or NaF (10 mM). Zinc sulfate was added in concentrations 12.5–100 µM. In additional experiments insulin (monocomponent porcine insulin Novo, 100 µU/ml) was added to membranes slightly stimulated by glucagon (0.56 or 5.6 nM). Total volume was 1.42 ml. Incubation was performed at 37 °C in an orbital water bath shaker, and the reaction was started by the addition of 50 µl of the liver membrane preparation. Final protein concentrations were 27–88 µg/ml. At 10 min a one ml sample was heated at 100 °C for 4 min. Samples were chromatographed on

7 x 40 mm AG 50W-X8, 100–200 mesh, hydrogen form, and cAMP-containing eluates were taken to dryness, dissolved in 0.1 M Na acetate buffer pH 4.0, and assayed for cAMP by the method of Gilman (1970).

Results and Discussion

The system produced a linear increase in cAMP during the 10 min incubation period. There was no measurable cAMP phosphodiesterase activity. Although all of the present experiments were performed with the above complete medium, it was subsequently found that the exclusion of ATP regeneration system as well as of dithiothreitol and EDTA did not materially affect the rate of cAMP synthesis. Zinc sulfate, at all concentrations tested, significantly inhibited glucagon-stimulated as well as fluoride-stimulated cAMP production (Fig. 1). Insulin exerted no influence.

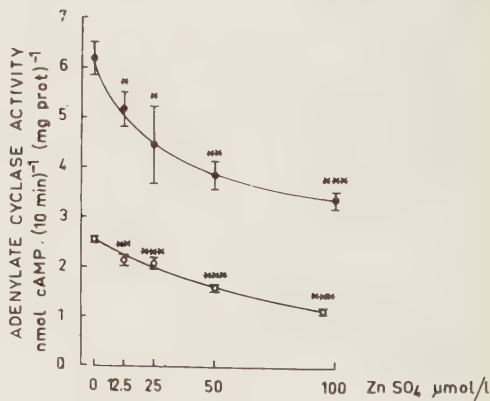


Fig. 1 Effects of zinc ions on adenylate cyclase activity in human liver membranes. The enzyme was stimulated by either glucagon (●) 56 nM or NaF (○) 10 mM. Means $\pm$ S.D. of 3 separate incubations. Differences from incubates without zinc ions: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ (Student's t test).

Received: 25 June 1978

Accepted: 7 June 1979

0018–5043/79 0932–0530 \$ 03.00 © 1979 Georg Thieme Publishers

An inhibitory effect of zinc ions on rat liver adenylate cyclase was found in early studies by *Rall and Sutherland* (1962). In the present human system the same influence was noted. The lowest concentration used, 12.5 μM , is within the human plasma zinc range. Inhibition was found whether glucagon or fluoride was used as activator. In contrast to glucagon, fluoride is thought to exert its stimulatory influence at the actual catalytic part of the enzyme system (*Birnbaumer, Pohl and Rodbell* 1973) or possibly at the transducer function (*Harwood and Rodbell* 1973) which couples hormone receptors to the catalytic moiety. Accordingly, it would seem probable that the zinc effect is exerted at one of these two sites, or both.

Zinc ions may be one of several factors in target cells that exert response-modulating action at hormonal stimulation.

Acknowledgements

The studies were supported by grants from the Medical Faculty, University of Lund, The Ernhold Lundström Foundation, The Swedish Society of Medical Sciences, and the Nordic Insulin Foundation.

Requests for reprints should be addressed to: Dr. J. Malmquist, Department of Medicine, Malmö General Hospital, S-21401 Malmö (Sweden)

References

- Birnbaumer, L., S.L. Phol, M. Rodbell:* J. Biol. Chem. 248: 1857–1860 (1971)
- Czech, M.P.:* Ann. Rev. Biochem. 46: 359–384 (1977)
- Exton, J.H., S.B. Lewis, R.J. Ho, G.A. Robison, C.R. Park:* Ann. N.Y. Acad. Sci. 185: 85–99 (1971)
- Gilman, A.G.:* Proc. Natl. Acad. Sci. U.S. 67: 305–312 (1970)
- Harwood, J.P., M. Rodbell:* J. Biol. Chem. 248: 4901–4904 (1973)
- Israelsson, B., A. Berglund, U. Ljungqvist, J. Malmquist:* Scand. J. Clin. Lab. Invest. 38: 289–293 (1978)
- Rall, T.W., E.W. Sutherland:* J. Biol. Chem. 238: 1228–1232 (1962)

Horm. Metab. Res. 11 (1979) 531–532

Effect of Estrogen on Heparin-Releasable Liver Lipases

J. Jubelin, G. Lam Van and J. Boyer

Metabolic Unit, Hôpital Conception, Marseille, France

We have recently shown (*Valette, Verine, Salers and Boyer* 1977) that ethinylestradiol (EE) reduced the levels of alkaline pH lipase in crude subcellular fractions from rat liver. By using isolated perfused rat liver preparations, we show in this paper that the fall in lipolytic activity involves a marked decrease in levels of two distinct heparin-releasable hepatic lipases.

Methods

Adult female rats (220–240 g) received EE (6 μg in 0.5 ml of 10% arabic) or gum arabic only (control animals) by stomach intubation daily for 21 days. They were then anesthetized (sodium pentobarbitone i.p., 80 mg/kg b.w.) and prepared for liver perfusion according to *Hems et al.* (1966). The perfusion medium consisted of 0.05% glucose and 3% serum albumin in Krebs bicarbonate buffer (pH 7.4) gassed with oxygen (95% with 5% CO_2) at 37 °C. The perfusion rate was 18 ml/min. Heparin (2 mg/kg b.w.) was infused within 10 s in 0.9% saline (1.0 ml) via the portal vein and then perfusion medium alone was passed through the liver for 3 min. Contiguous samples of liver effluent were collected, frozen and stored at –20 °C. Triester lipase (TEL) was assayed at 37 °C in a volume of 1 ml containing: 0.1 ml Tris-HCl, 0.25% defatted albumin, 1 mM tri- $[\text{^3H}]$ oleoylglycerol (pH 8.0). The assay system for monoester lipase (MEL) contained: 0.1 mM Tris-HCl, 0.5% defatted albumin, 1 mM $[\text{^3H}]$ oleoylethanol in a volume of 1 ml at 37 °C (pH 8.5).

The release of $[\text{^3H}]$ oleic acid over the 15 min-period of assay was monitored as reported (*Arnaud and Boyer* 1976). One unit of lipase activity corresponds to the release of 1 μmole of acid per min.

Results and Discussion

No lipolytic activity was detectable in effluent before infusion of heparin. As shown in Fig. 1 the peaks of TEL and MEL activities were released about 20 s after the 10 s pulse of heparin in both normal and EE-treated rats. In treated rats, total TEL and MEL activities corresponded each to 1.3 unit of activity, i.e. 31 and 29% of those assayed in control rats (4.3 and 4.6 units, respectively). The ratio of liver weight: body weight was slightly increased in treated rats (3.7 compared to 3.4% in control rats). The results show that EE markedly reduced the levels of lipases releasable by heparin, i.e. supported by the liver cell membrane (*Arnaud and Boyer* 1976). They supply conclusive support to the observation by *Applebaum, Goldberg, Pykalistö, Brunzell and Hazzard* (1977) that decline in post-heparin lipolytic activity in blood from humans under estrogens is due to decreased hepatic lipases. These enzymic changes may play a role in the estrogen-induced increase in hepatic production of triglyceride-rich very low density lipoproteins leading to hypertriglyceridemia (*Weinstein, Seedman and Veldhuis* 1975).

Received: 30 July 1978

Accepted: 7 June 1979

0018–5043/79 0932–0531 \$ 03.00 © 1979 Georg Thieme Publishers

Adenylate cyclase activity in human liver membranes correlated to insulin release and glucose tolerance

BO ISRAELSSON

Department of Medicine, University of Lund, Malmö General Hospital, S-214 01 Malmö, Sweden

Israelsson, B. Adenylate cyclase activity in human liver membranes correlated to insulin release and glucose tolerance. *Scand. J. clin. Lab. Invest.* **40**, 159–162, 1980.

Glucagon-stimulated adenylate cyclase activity has been studied in human crude liver membranes prepared from liver biopsies taken at cholecystectomies. Enzyme activities were negatively correlated to 0–40 min plasma insulin increments determined preoperatively by oral glucose tolerance tests.

Key-words: Adenylate cyclase activity; glucose tolerance test; human liver membranes; insulin secretion

Bo Israelsson, M.D., Department of Medicine, Malmö General Hospital, S-214 01 Malmö, Sweden

Glucagon has a regulatory effect in glucose metabolism in the liver through activation of adenylate cyclase in the plasma membranes of hepatocytes [4]. In contrast to the established role of cyclic adenosine monophosphate (cyclic AMP, cAMP) as the second messenger for glucagon action, no signal mechanism for insulin has been securely identified. However, insulin has an inhibitory effect on cAMP production in the liver in experiments with isolated hepatocytes [14] or perfused livers [3]. Data concerning the mechanism for this effect are conflicting [12].

The present study concerns the correlation between glucagon stimulated adenylate cyclase activity in human liver membranes and preoperatively determined glucose tolerance and insulin release.

MATERIAL AND METHODS

Subjects and procedures. Individuals, healthy apart from gall-stone disease and on the waiting list for operation, were asked to participate in the experiments. Those who gave their consent to liver biopsy, after information on the purpose and possible risks, were invited to a preoperative investigation including an oral glucose tolerance test (30 g glucose/m² body surface area) with determinations of blood glucose and plasma insulin (at 0, 40, and 120 min). With the exception of three individuals, biopsies were taken within 2 months from the preoperative tests. In the interval no dietary regime was prescribed and the maximum body weight change was 2.6 kg. Details are presented in Table I.

Chemicals. Protein kinase, protein kinase inhibi-

TABLE I. Glucose tolerance and hepatic adenylate cyclase activity in seventeen healthy subjects.

Subjects	Age	Sex	Actual/ideal weight	Days between preoperative test and operation	Blood glucose (fasting) (mmol/l)	Blood glucose (120 min) (mmol/l)	Plasma-insulin (40 min fasting) (mIU/l)	nmol cAMP/10 min $\times$ mg protein	Number of relatives with diabetes
1	40	F	1.22	34	4.8	6.0	31	1.94	1
2	54	F	0.74	27	5.4	8.8	42	2.26	
3	47	M	1.13	41	5.1	8.0	46	2.28	
4	56	F	0.83	25	4.4	5.4	47	4.01	1
5	60	F	1.07	22	4.6	8.4	50	2.75	
6	44	F	0.87	28	4.4	3.8	50	1.84	1
7	44	F	1.25	294	5.2	7.6	55	1.86	1
8	29	F	0.86	59	4.2	5.9	63	2.14	1
9	60	F	0.97	28	4.9	5.9	66	3.62	4
10	54	M	1.10	27	5.4	3.9	67	1.34	
11	47	F	1.41	175	5.1	5.9	69	1.76	
12	38	F	1.09	32	4.2	5.6	76	1.41	
13	42	F	1.09	21	4.7	6.6	77	2.05	
14	26	F	0.80	174	3.9	5.3	88	1.33	
15	25	F	0.97	20	3.6	4.8	92	1.43	1
16	50	F	1.16	64	5.2	4.2	96	2.34	
17	49	F	1.00	15	4.6	4.0	158	1.21	

tor, cyclic AMP, and adenosine 5'-triphosphate (ATP) were obtained from Sigma Chemical Co., 1-methyl, 3-isobutylxanthine was from Aldrich Chemical GmbH, Steinheim, G.F.R. Porcine glucagon was from Novo Industries, Copenhagen, Denmark. Dowex AG 50W-X8 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. [^3H]labelled cAMP was obtained from New England Nuclear, Boston, MA, U.S.A., and guanylyl 5'-imidodiphosphate (GMP-PNP) from Boehringer Mannheim, G.F.R.

Liver biopsy and crude membrane preparation. The methods for taking liver biopsies and for the preparation of liver homogenate and crude membranes are described elsewhere [7].

Incubation conditions. The incubation medium contained 50 mmol/l Tris-HCl, pH 7.6, 1 mmol/l 1-methyl, 3-isobutylxanthine, 4 mmol/l MnCl_2 , 4 mmol/l ATP and 0.1 mmol/l GMP-PNP. To 1.25 ml of medium, glucagon was added to a final concentration of 56 nmol/l. Incubation was performed at 37°C in a shaking water bath and the reaction was started by the addition of 50 μl of liver membrane preparation giving a final protein concentration of 39–132 $\mu\text{g}/\text{ml}$. Total volume was 1.32 ml. At 10 min a 1 ml sample was taken and heated at 100°C for 4 min. Zero time samples were obtained by adding membrane preparation to medium at 100°C.

Assay methods. Cyclic AMP was assayed as described elsewhere [7]. Zero time values were based on duplicate samples and 10 min values on triplicate samples. Blood glucose was determined with glucose oxidase according to Marks [13]. Plasma insulin was measured by radioimmunoassay according to Thorell & Larson [15]. Protein was determined by the Hartree modification [5] of the Lowry method, with human serum albumin as standard. Data were analysed by linear regression.

RESULTS

Preoperative tests were performed and liver biopsies were taken from seventeen subjects without any complications. Five subjects had

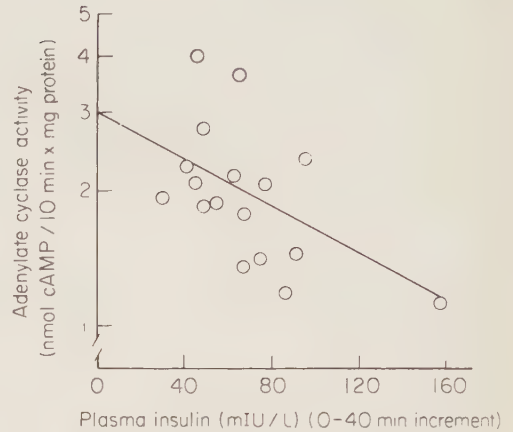


FIG. 1. Relationship between insulin secretion at oral glucose tolerance test and hepatic adenylate cyclase activity (logarithmic scale). The regression line has been drawn. $n = 17$, $r = -0.52$, $P < 0.05$.

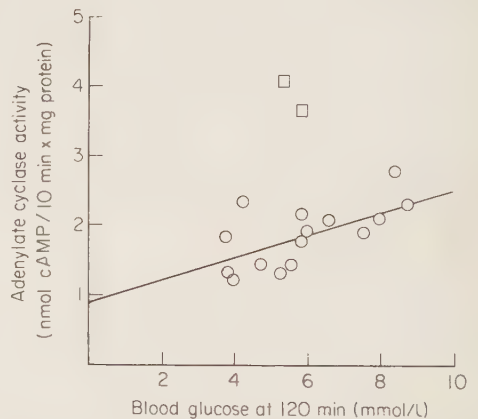


FIG. 2. Relationship between blood glucose values at 120 min in oral glucose tolerance tests and hepatic adenylate cyclase activities. Results indicated by the two open squares ($\square$) were excluded in regression analysis. The regression line has been drawn. $n = 15$, $r = 0.62$, $P < 0.05$.

abnormally high blood glucose (> 6.5 mmol/l) at 120 min in the oral glucose tolerance test. Six patients had heredity for diabetes (Table I).

Insulin release expressed as 0–40 min plasma insulin increments were negatively correlated ($r = -0.52$, $P < 0.05$) to the logarithms of liver membrane adenylate cyclase activities (Fig. 1). In contrast neither the sum nor the individual values at 0, 40, and 120 min had any significant correlation to adenylate cyclase activity.

Interdependence between the blood glucose values at 120 min and the adenylate cyclase activities were evaluated by linear regression analysis. For unknown reasons two subjects had considerably higher adenylate cyclase activity than the others. Calculations without these two subjects gave a positive correlation between blood glucose at 120 min and adenylate cyclase activity ($r = 0.62$, $P < 0.05$). Including these two subjects there was no such correlation (Fig. 2). Fasting or 40 min blood glucose values were not correlated to adenylate cyclase activity.

DISCUSSION

Previously cAMP production in human liver has only been investigated with *in vivo* techniques [1, 11]. Because of the multitude of factors modulating adenylate cyclase activity and since the influence of phosphodiesterase activity can not be excluded, interpretations of results from such studies are rather difficult. In the present *in vitro* study, possible hepatic effect of anesthetic agents could not be avoided. Because of the risk for liver toxicity halothane was not used [2]. On the other hand, barbiturate is not quite ideal since it may have some influence on cAMP levels in the liver [9]. Protracted procedures needed to obtain purified liver membranes may lead to disturbances of enzyme activity [8]. Therefore in this study a simple method was used for the preparation of liver specimens [7].

Glucose tolerance expressed as blood glucose at 120 min was not significantly correlated to adenylate cyclase activity. Only if two subjects with outstandingly high enzyme activity were excluded did a positive correlation occur (Fig. 2).

Opinions differ on the ability of insulin to modulate adenylate cyclase in broken cell systems [12]. Recently a system with hepatocyte plasma membranes was described where adenosine was of critical importance for insulin effect on the adenylate cyclase system [10].

This study has demonstrated a weak inverse relation between glucose-induced insulin secretion and hepatic cAMP production. This is in accordance with previous data of Hepp [6] and points to the possibility that insulin exerts an enduring inhibitory influence on the glucagon responsiveness of hepatic adenylate cyclase.

REFERENCES

- 1 Bomboy, J.D., Jr, Lewis, S.B., Lacy, W.W., Sinclair-Smith, B.C. & Liljenquist, J.E. Transient stimulatory effect of sustained hyperglucagonemia on splanchnic glucose production in normal and diabetic man. *Diabetes*, **26**, 177, 1977.
- 2 Brown, B.R. & Sipes, I.G. Biotransformation and hepatotoxicity of halothane. *Biochem. Pharmacol.* **26**, 2091, 1977.
- 3 Exton, J.H., Lewis, S.B., Ho, R.J., Robison, G.A. & Park, C.R. The role of cyclic AMP in the interaction of glucagon and insulin in the control of liver metabolism. *Am. N.Y. Acad. Sci.* **185**, 85, 1971.
- 4 Exton, J.H., Robison, G.A., Sutherland, E.W. & Park, C.R. Studies on the role of adenosine 3',5'-monophosphate in the hepatic actions of glucagon and catecholamines. *J. biol. Chem.* **246**, 6166, 1971.
- 5 Hartree, E.F. Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Analyt. Biochem.* **48**, 422, 1972.
- 6 Hepp, K.D. Adenylate cyclase and insulin action. Effect of insulin, nonsuppressible insulin-like material, and diabetes on adenylate cyclase activity in mouse liver. *Eur. J. Biochem.* **31**, 266, 1972.
- 7 Israelsson, B., Berglund, A., Ljungqvist, U. & Malmquist, J. Adenylate cyclase activity in human liver membranes and its inhibition by adenosine and adenine nucleotides. *Scand. J. clin. Lab. Invest.* **38**, 289, 1978.
- 8 Katz, M.S., Kalish, M.I., Piñeyro, M.A. & Gregerman, R.I. Quantitation of epinephrine- and glucagon-sensitive adenylate cyclase of rat liver. Implications of alterations of enzymatic activities during preparation of particulate fractions and membranes. *Biochim. biophys. Acta*, **540**, 205, 1978.
- 9 Kimura, H., Thomas, E. & Murad, F. Effects of decapitation, ether and pentobarbital on guanosine 3',5'-phosphate and adenosine 3',5'-phosphate levels in rat tissues. *Biochem. biophys. Acta*, **343**, 519, 1974.
- 10 Kiss, Z. Inhibition of the glucagon stimulated adenylate cyclase activity by insulin. *FEBS Lett.* **92**, 29, 1978.
- 11 Liljenquist, J.E., Bomboy, J.D., Lewis, S.B., Sinclair-Smith, B.C., Felts, P.W., Lacy, W.W., Crofford, O.B. & Liddle, G.W. Effect of glucagon on net splanchnic cyclic AMP production in normal and diabetic men. *J. clin. Invest.* **53**, 198, 1974.
- 12 Loten, E.G., Assimacopoulos-Jeannet, F.D., Exton, J.H. & Park, C.R. Stimulation of a low K_m phosphodiesterase from liver by insulin and glucagon. *J. biol. Chem.* **253**, 746, 1978.
- 13 Marks, V. An improved glucose-oxidase method for determining blood, c.s.f. and urine glucose levels. *Clin. chim. Acta*, **4**, 395, 1959.
- 14 Pilkis, S.J., Claus, T.H., Johnson, R.A. & Park, C.R. Hormonal control of cyclic 3':5'-AMP levels and gluconeogenesis in isolated hepatocytes from fed rats. *J. biol. Chem.* **250**, 6328, 1975.
- 15 Thorell, J.I. & Larson, S.M. Insulin, p. 205 in *Radioimmunoassay and related techniques*. C.V. Mosby, St Louis, 1978.

Received 26 April 1979

Accepted 30 August 1979

Changes in adenylate cyclase and 5'-nucleotidase activities in liver membranes from alloxan diabetic rats

B. Israelsson and I. Tengrup

Departments of Medicine and Surgery, University of Lund, Malmö General Hospital, S-21401 Malmö (Sweden), 26 April 1979

Summary. Liver membrane adenylate cyclase activity was significantly higher and 5'-nucleotidase activity significantly lower in alloxan diabetic rats compared with normal rats.

The enzyme 5'-nucleotidase (EC3.1.3.31) is located in the external part of the plasma membrane. Adenylate cyclase (EC4.6.1.1.) is situated in the interior part of the plasma membrane. The hepatic level of adenosine 3'5'-monophosphate (cyclic AMP, cAMP), the product of adenylate cyclase, is increased in alloxan diabetic rats¹. Adenosine,

the product of 5'-nucleotidase, is rapidly translocated from the outside into cells where it is metabolized². This causes steady state levels to be very low. In previous studies adenosine has been linked with insulin action³ and has also been found to have a strong inhibitory influence on adenylate cyclase activity in liver membranes⁴. Recently, recipi-

cal alterations in 5'-nucleotidase and adenylate cyclase activities were found during cartilage maturation². The present study concerns the effect of alloxan-induced insulinopenia in rats on the activities of hepatic 5'-nucleotidase and adenylate cyclase. The study was also performed on rats investigated concerning the effect of zinc on wound healing. The results of this latter investigation are to be presented elsewhere.

Materials and methods. 35 male Sprague-Dawley rats weighing 180–230 g were used. They received standard rat chow (Astra-Ewos, Sweden) and had free access to drinking water. 17 animals were made diabetic by rapid i.v. injection of alloxan monohydrate (40 mg/kg) in 0.9% saline. Blood glucose above 7.8 mmol/l combined with glucosuria (Clinistix® positive) indicated diabetes. Because of the simultaneous wound healing study all the animals, diabetics and controls, were operated with a piece of sponge placed s.c. 14 days after alloxan treatment. Directly thereafter 10 rats were injected with 75 µg ZnAc in a single i.m. dose while 8 rats were injected with the same dose for 4 days. All animals were sacrificed on the same day. The rats were killed with ether. The abdominal and chest cavities were opened and 5 ml of blood was drawn from the right heart chamber whereupon 50 ml of 0.9% saline was injected into the left heart chamber. The liver was removed and about 1 cm³ was taken for enzyme studies.

The method for preparation of a crude membrane fraction was the same as that presented elsewhere⁴ except that the liver homogenates were frozen in liquid nitrogen before further preparation. The incubation medium contained 50 mmol/l Tris-HCl, pH 7.6, 1 mmol/l 1-methyl, 3-isobutyl-xanthine, 4 mmol/l MnCl₂, 4 mmol/l ATP and 0.1 mmol/l GMP-PNP. To 1.25 ml of medium was added glucagon (final concentration 56 nmol/l). Incubation was performed at 37 °C in a shaking water bath and the reaction was started by the addition of 50 µl of liver membrane

preparation giving a final protein concentration of 39–132 µg/ml. Total volume was 1.32 ml. At 10 min a 1-ml sample was taken and heated at 100 °C for 4 min. Zero time samples were obtained by adding membrane preparation to medium at 100 °C. Cyclic AMP was determined according to a method presented elsewhere⁴. Protein and glucose were measured by the techniques of Hartree and Marks, respectively^{6,7}. 5'-nucleotidase was measured spectrophotometrically according to Belfield and Goldberg⁸.

Results. The activity of 5'-nucleotidase in diabetic rats was significantly lower, 50.3 ± 17.8 milliunits/mg protein, than in controls, 65.7 ± 10.2 milliunits/mg protein (*p* < 0.01). The reverse was true for the adenylate cyclase activity with 3.14 ± 0.57 and 2.76 ± 0.42 nmol cAMP (mg protein)⁻¹ (10 min)⁻¹ for the diabetics and controls, respectively (*p* < 0.05) (table). The addition of alloxan (1 mmol/l) to the incubation media was found not to affect enzyme activities. Taking all observations from diabetic and control animals together there was a significant inverse correlation between 5'-nucleotidase and adenylate cyclase activities (*r* = -0.46, *p* < 0.01) (figure). Zinc treatment did not have any influence on zinc concentration in hepatic tissue or on the 2 enzyme activities studied.

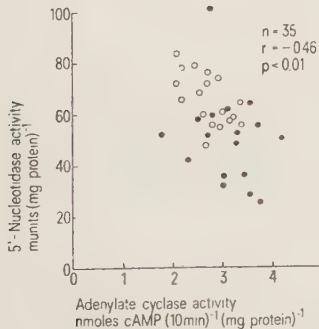
Discussion. Adenosine, the product of 5'-nucleotidase, is a potent inhibitor of adenylate cyclase activity in the liver¹. The pronounced decrease in 5'-nucleotidase activity and the increase in adenylate cyclase activity in the diabetic rats may be interrelated through adenosine levels. Reciprocal alterations in these enzyme activities were recently found also in cartilage during maturation², and may have been the effect of insulin-like factors of the somatomedin group. Bhatena et al. recently found liver 5'-nucleotidase activity to be considerably lower in diabetic rats compared with controls⁹. This connection between the activity of 5'-nucleotidase and the diabetic state of streptozotocin treated rats was also recognized by Soman and Felig¹⁰, who furthermore found the enzyme activity to be influenced by insulin supplementation. They also proposed that the increased glucagon stimutable adenylate cyclase activity of diabetic animals might be an effect of increased glucagon binding, which is in conflict with the results of others^{9,11} on down-regulation of glucagon receptors in the hyperglucagonemia of insulin deficient states.

In conclusion, alloxan diabetes is associated with a decrease in hepatic 5'-nucleotidase activity and an inversely proportional increase in adenylate cyclase activity. These changes may possibly be interrelated through adenosine levels. It remains to be elucidated whether these enzyme perturbations are related to insulin deficiency or to some other aspect of the diabetic state.

Hepatic membrane enzyme activities in diabetic and control animals

	Diabetics	Controls	<i>p</i>
Adenylate cyclase activity [nmol cAMP (10 min) ⁻¹ (mg protein) ⁻¹]	3.14 ± 0.57	2.76 ± 0.42	< 0.05
5'-nucleotidase activity (munits/mg protein)	50.3 ± 17.8	65.7 ± 10.2	< 0.01
<i>n</i>	17	18	

Mean ± SD.



Relationship between hepatic 5'-nucleotidase and adenylate cyclase activities in diabetic (●) and control (○) animals.

- 1 S. J. Pilkis, J. H. Exton, R. A. Johnson and C. R. Park, *Biochim. biophys. Acta* 343, 250 (1974).
- 2 J. Schrader, R. M. Berne and R. Rubio, *Am. J. Physiol.* 223, 159 (1972).
- 3 Z. Kiss, *FEBS Lett.* 92, 29 (1978).
- 4 B. Israelsson, A. Berglund, U. Ljungqvist and J. Malmquist, *Scand. J. clin. Lab. Invest.* 38, 289 (1978).
- 5 G. A. Rodan, L. A. Bourret and L. S. Cutler, *J. Cell Biol.* 72, 493 (1977).
- 6 E. F. Hartree, *Analyt. Biochem.* 48, 422 (1972).
- 7 V. Marks, *Clin. chim. Acta* 4, 395 (1959).
- 8 A. Belfield and D. M. Goldberg, *Clin. Chem.* 15, 931 (1969).
- 9 S. J. Bhatena, N. R. Voyles, S. Smith and L. Recant, *J. clin. Invest.* 61, 1488 (1978).
- 10 V. Soman and P. Felig, *J. clin. Invest.* 61, 552 (1978).
- 11 C. B. Srikant, D. Freeman, K. McCorkle and R. H. Unger, *J. biol. Chem.* 252, 7434 (1977).

CYCLIC AMP ACCUMULATION IN HUMAN FIBROBLAST CULTURES: NORMALS VERSUS DIABETICS

B. Israelsson, J. Malmquist, J. Flink, and T. Kjellström

Department of Medicine, Malmö General Hospital,
University of Lund, S-214 01 Malmö, Sweden

Summary

Skin fibroblasts from four subjects with maturity onset diabetes and six normals were studied. Isoproterenol stimulated cyclic AMP accumulation was determined using a prelabelling technique with [^3H]adenine. Fibroblasts from diabetics exhibited cyclic AMP values significantly higher than cells from normals.

Subjects with diabetes mellitus have a decreased life expectancy. Studies on skin fibroblasts from diabetics have pointed to a premature aging (1). After adrenaline stimulation senescent fibroblasts increase their intracellular cyclic AMP more than young cells (2). In order to further elucidate mechanisms of the metabolic disturbance in diabetics the present study deals with cyclic AMP accumulation in fibroblast cultures from normal and diabetic subjects.

Material and Methods

Skin fibroblasts were cultured from punch biopsies taken from the medial aspect of the left upper arm of six healthy subjects aged 30-50 and six subjects 49-71 years old with maturity onset diabetes. Cells were grown in humidified air at 37°C in Eagle's minimal essential medium (MEM) containing 10% fetal calf serum, 2% L-glutamine, 1% non-essential amino acids, and 0.2% gentamicin. The same serum batch was used in all experiments. Cells at passage 2-6 were used, i.e., cells at an average of 4-12 population doublings in vitro. The fibroblasts were cultured in 3 cm dishes in 2 ml growth medium. Cells were allowed to settle for 48 h. Subsequently medium was changed every 24 h. Studies were made on the 3-6th day after subcultivation.

Cyclic AMP accumulation was determined with a prelabelling technique using [^3H]adenine (Amersham) for subsequent determinations of [^3H]cyclic AMP (3). Immediately after the growth medium had been poured off 3 ml MEM containing 30 μCi [^3H]adenine was added to each dish. Attempts were made to make concomitant determinations of 5'-nucleotidase activity according to Belfield and Goldberg (4). For that reason adenosine deaminase, adenosine monophosphate, and glycerophosphate were added. These results will be presented elsewhere. After 60 min of prelabelling cells were washed twice with 3 ml MEM and finally with another 3 ml MEM for 20 min at 37°C. Cells were then incubated for 10 min with a medium which contained MEM supplemented with 10^{-3} mol/l cyclic AMP (Sigma Chemical Co), 1.2×10^{-5} mol/l isoproterenol, 10^{-3} mol/l thiourea, and 10^{-4} mol/l ascorbate. Thiourea and ascorbate were included to protect against catecholamine oxidation (5). Incubations were

terminated after 10 min by transferring the medium to tubes containing 0.1 ml 2% sodium dodecyl sulfate. [^3H]cyclic AMP liberated by the fibroblasts into the medium was determined according to Solomon et al (6). Aquasol-2 was used for liquid scintillation at 10°C . Cyclic AMP was expressed as disintegrations per minute (DPM) per mg protein after individual recovery and quench corrections.

Protein was determined with the Fluram method (7) using serum albumin as standard. Cells were scraped out of the dishes in H_2O with a rubber policeman and carefully disintegrated with a motor-driven micro glass homogenizer, or dissolved in situ with 0.2 mol/l NaOH for 15 min at 37°C .

Results

Up to a certain level of cell density (150 μg protein/dish) cyclic AMP accumulation per mg protein increased in parallel with protein. Fibroblasts grown more densely (100-450 μg protein/dish) produced cyclic AMP per mg protein at a more constant level. However, cells from maturity onset diabetes were generally more active than those from controls (Fig 1).

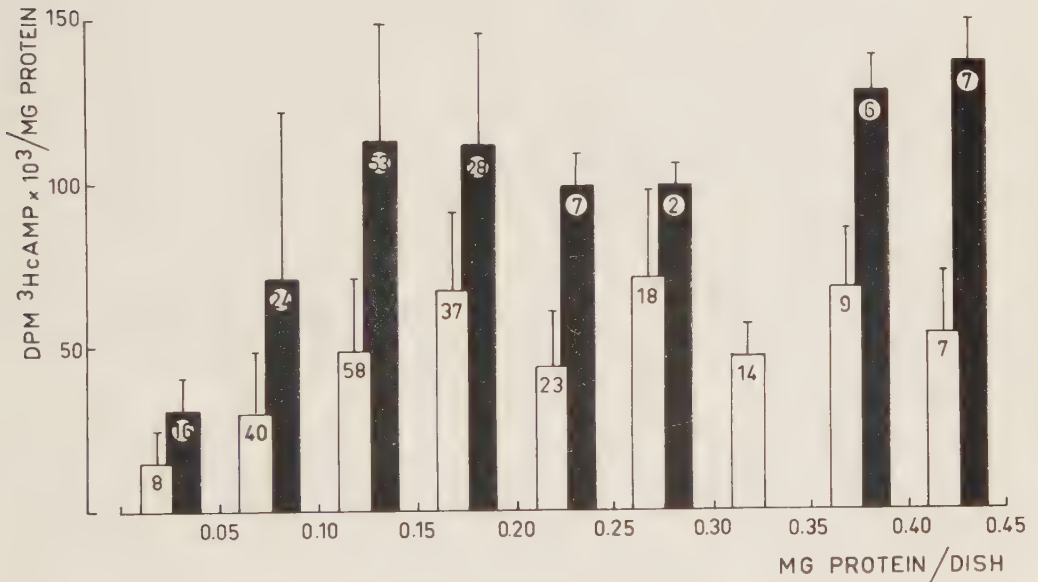


FIG. 1

Isoproterenol stimulated (1.2×10^{-5} mol/l) cyclic AMP accumulation in fibroblast cultures from six normals (open bars) and six diabetics (black bars). Number of observations are indicated.

The diabetic subjects were 49-71 years old and the normals were in the age of 30-50. The interrelation between donor age and cyclic AMP liberated from the fibroblasts is shown in Figure 2. In neither of the two groups was there any correlation between nucleotide accumulation and donor age.

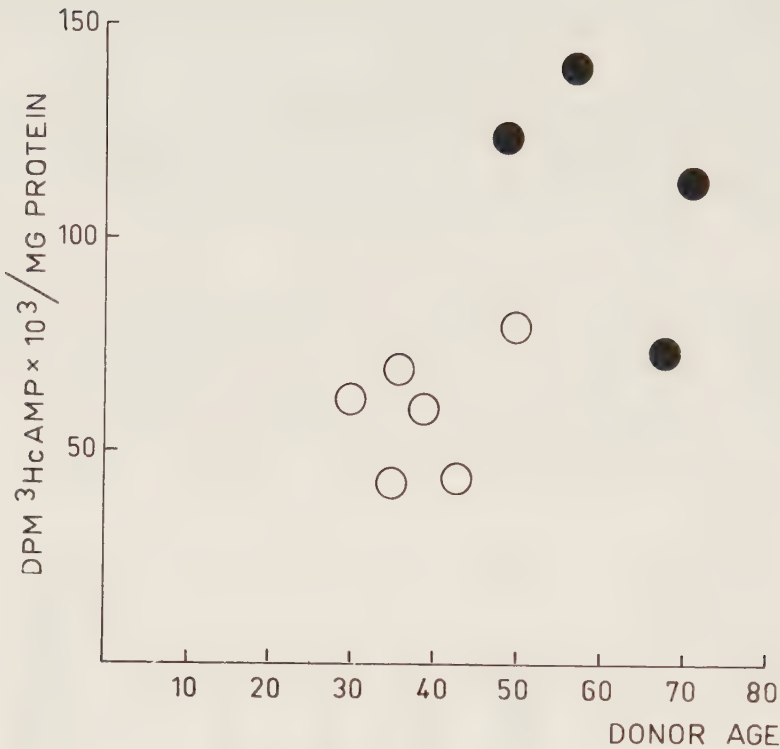


FIG. 2

Isoproterenol stimulated (1.2×10^{-5} mol/l) cyclic AMP accumulation in fibroblast cultures (100-450 μ g protein/dish) related to donor age of normals (○) and diabetics (●). Each point represents the mean of 17-57 observations.

In the range of constant cyclic AMP accumulation per mg protein (100-450 μ g protein/dish) totally 269 observations were made on fibroblasts from four diabetics and six normals. Cells from the diabetic subjects displayed nucleotide levels significantly higher, $(112 \pm 28) \times 10^3$, than those exhibited by the normal cells, $(59 \pm 14) \times 10^3$ (Table I).

Discussion

In the present study differences in cyclic AMP accumulation could be the result of alterations in adenylate cyclase or phosphodiesterase activity. Phosphodiesterase inhibitor was not used in order to avoid restriction of nucleotide efflux from cells (8).

Strict comparison of net cyclic AMP release by the [³H]adenine prelabelling method requires that uptake of tracer is the same in both cell groups and that cell content of ATP is likewise similar. Thus, the differences in [³H]cyclic AMP might possibly be related to these factors rather than to differences in actual synthesis and/or hydrolysis of cyclic AMP. However, this would still mean that a difference in nucleotide metabolism exists between the two fibroblast groups.

Previous studies on replicative life-span of fibroblasts have pointed to a mean decrement of 0.25 mean population doublings in vitro for each year of donor age (9). However, the correlation between age and population doublings was weak. It is therefore not surprising that we found no age-related variations in cyclic AMP accumulation within the groups of fibroblasts from normals and diabetics. However, in spite of the small number of subjects investigated (n=10) the group difference in cyclic AMP accumulation was highly significant ($p < 0.005$). These observations make it probable that studies on cyclic AMP accumulation represent a method to illustrate a metabolic disturbance in fibroblasts from diabetics.

TABLE I

Isoproterenol stimulated cyclic AMP accumulation (mean $\pm$ SD) in fibroblast cultures from normal and diabetic subjects

Donor	Sex	Age	Diabetes duration (years)	Culture passage No.	DPM [3 H]cAMP $\times 10^3$ mg protein	Group mean
MOD	M	49	5	6	123 $\pm$ 18 (n=19)	112 $\pm$ 28
MOD	M	57	3	2,4	139 $\pm$ 26 (n=32)	
MOD	M	68	14	3	73 $\pm$ 15 (n=23)	
MOD	F	71	8	3,4	114 $\pm$ 25 (n=29)	
N	M	30	-	2,3	62 $\pm$ 18 (n=36)	59 $\pm$ 14
N	M	35	-	4,6	43 $\pm$ 15 (n=57)	
N	M	36	-	2	69 $\pm$ 9 (n=17)	
N	M	39	-	3,4	60 $\pm$ 44 (n=17)	
N	M	43	-	3	44 $\pm$ 6 (n=20)	
N	M	50	-	6	79 $\pm$ 19 (n=19)	

Difference between groups significant by Student's t test ($p < 0.005$) as well as by Mann-Whitney rank sum test ($p = 0.02$)

MOD = maturity onset diabetics. N = normals

Acknowledgements

This work was supported by grants from the Swedish Medical Research Council (grant No B81-19X-05702-02), the Medical Faculty, University of Lund, and the Ernhold Lundström Foundation,

References

1. S. GOLDSTEIN, J. Invest. Dermatol. 73 19-23 (1979).
2. R.J. HASLAM and S. GOLDSTEIN, Biochem. J. 144 253-263 (1974).
3. R.W. BUTCHER, J. Cycl. Nucl. Res. 4 411-421 (1978).
4. A. BELFIELD and D.M. GOLDBERG, Clin. Chem. 15 931-939 (1969).
5. R. BARBER, L.A. KELLY, R.F. MCGUIRE, and R.W. BUTCHER, J. Cycl. Nucl. Res. 3 249-261 (1977).
6. Y. SOLOMON, C. LONDOS, and M. RODBELL, Analyt. Biochem. 58 541-548 (1974).
7. P. BÜHLEN, S. STEIN, W. DAIRMAN, and S. UDENFRIEND, Arch. Biochem. Biophys. 155 213-220 (1973).
8. L.A. KELLY, C. WU, and R.W. BUTCHER, J. Cycl. Nucl. Res. 4 423-435 (1978).
9. S. GOLDSTEIN, E.J. MOERMAN, J.S. SOELDNER, R.E. GLEASON, and D.M. BARNETT, Science 199 781-782 (1978).

